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MODULATION OF EFFLUX OF XENOBIOTICS IN MULTIDRUG RESISTANT (MDR) CANCER CELL LINES BY PLURONIC® BLOCK COPOLYMERS

By

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EFFET DES COPOLYMÈRES EN BLOC DE LA FAMILLE DES PLURONICS® COMME MODULATEUR DE L'EFFLUX DE XÉNOBIOTIQUES DANS LES LIGNÉES CELLULAIRES CANCÉREUSES EXPRIMANT UN PHÉNOTYPE DE RÉSISTANCE AUX DROGUES MULTIPLES (MDR)

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This thesis is entirely dedicated to my beloved father Avedis Colakyan who lost his fight to stage (IV) larynx cancer on January 27, 2003 at the age of 48 years.

"Conducting this thesis has brought back memories of our everyday battle with this disease. I am still continuing your fight and hoping that one-day, I can make an important contribution to this field."

Klodia Colakyan

Résumé

L'émergence de la résistance aux drogues multiples (*Multi Drug Resistance*, MDR) médiée par la surexpression de plusieurs protéines liant l'ATP (*ATP-Binding Cassette*, ABC) constitue un obstacle majeur qui limite souvent le succès des traitements ciblant certains cancers et autres maladies. Trois transporteurs ABC cliniquement représentatifs qui sont associés au MDR sont : la protéine de perméabilité (*Permeability Protein,* P-gp), la protéine de résistance aux drogues multiples (*Multidrug Resistant Protein* 1, MRP1), et la protéine mammaire de résistance cancéreuse (*Breast Cancer Resistant Protein*, BCRP). Ces transporteurs fonctionnent comme des "*pompes à efflux*" transportant les xénobiotiques à l'extérieur de la cellule. La P-gp est aussi responsable du faible transport d'un grand nombre de composés de la lumière intestinale à la circulation sanguine. Conséquemment, la modulation de leurs activités peut être essentielle pour le succès des thérapies de plusieurs maladies, plus spécifiquement celles impliquant une chimiothérapie. Plusieurs modulateurs des systèmes d'efflux ont déjà été testés. Un type de modulateur sont les copolymères amphiphiliques en bloc (poloxamers) de la famille des Pluronics® et qui représenteront un intérêt particulier dans notre étude pour leur composition potentielle dans la formulation de drogues. Les Pluronics® sont composés de poly(éthylène oxide)(PEO) et de poly(propylène oxide) (PPO), ayant une structure $(PEO)_x(PPO)_y(PEO)_x$ (Schmolka, 1977). Des études *in vitro* et *in vivo* précédentes ont démontré l'habileté des Pluronics® à sensibiliser des cellules cancéreuses MDR surexprimant la P-gp (Alakhov et *al.*, 1996; Venne et *al.*, 1996; Batrakova et *al.*, 1996; Batrakova et *al.* 2003). Dans d'autres études utilisant des cellules transfectées avec MRP1 et MRP2 (cellules *Madin Darby Canine Kidney* (MDCKII), et des cellules cancéreuses de poumon résistantes à la doxorubicine et surexprimant MRP1 (cellules COR-L23\R), il a été démontré que le Pluronic® P85 augmente l'accumulation de vinblastine et de doxorubicine. Cependant, le Pluronic® P85 n'a montré aucun effet d'augmentation de cytotoxicité de la doxorubicine dans les cellules résistantes COR-L23/R (Batrakova et *al.*, 2003).

L'objectif général de notre étude est d'examiner *in vitro* l'effet des Pluronics® sur la fonction transporteur de trois protéines du type ABC impliquée dans la résistance aux drogues multiples: P-gp, BCRP et MRP1. Plus spécifiquement, au départ 21 copolymères ont été testés pour la détermination de la concentration critique de micellisation (CMC),

une des propriétés de base des surfactants. Également, le niveau de leur activité à induire l'accumulation du substrat rhodamine 123 (R123) dans les cellules cancéreuses mammaires (MCF-7 ADR) surexprimant la P-gp a été caractérisé. Ensuite les Pluronics® L61 et P85 ont été sélectionnés pour d'autres études afin de tester leur niveau inhibiteur à moduler l'activité des transporteurs membranaire P-gp, MRP1 et, pour la première fois, la BCRP. Pour chaque transporteur, les études absorption et de cytotoxicité ont été effectuées sur les cellules cancéreuses mammaires résistantes à la doxorubicine (MCF-7 ADR) surexprimant la P-gp, et les cellules leucémiques résistantes à la doxorubicine (HL-60 ADR) surexprimant la MRP1. Les cellules cancéreuses mammaires résistantes au topotecan (MCF-7 TPT) surexprimant la BCRP ont été sélectionnées progressivement à partir des cellules MCF-7 parental exposées au topotecan hydrochloride (TPT). Les études d'accumulation ont examiné l'influence des Pluronics® et d'autres inhibiteurs d'efflux (cyclosporine A, vérapamil, 17 ß-estradiol, GF120918) sur l'accumulation intracellulaire de différentes sondes comme substrat (rhodamine 123, fluorescéine, doxorubicine, mitoxantrone, topotecan). De plus, l'activité inhibitrice des Pluronics® a été comparée aux autres modulateurs d'efflux mentionnés ci-haut. Également, les tests de cytotoxicité ont déterminé la sensibilisation des cellules résistantes aux drogues cytotoxiques en présence du Pluronic® L61. Pour cela, les cellules ont été exposées à un traitement de drogues-polymères et la viabilité des cellules a été examinée à l'aide d'un test standard de XTT.

Les études d'accumulation ont démontré que les cellules MCF-7 ADR surexprimant la P-gp ont répondu le plus aux effets inhibiteurs des Pluronics®. Les polymères hydrophobiques comme les Pluronics® L92, L61 et P123 à leur concentration maximale d'efficacité ont augmenté l'accumulation de R123 dans les cellules MCF-7 ADR, davantage que Pluronic® P85, le polymère moins hydrophobe. Les Pluronics® ont démontré une efficacité limitée pour l'inhibition de l'efflux médié par la BCRP et le MRP1. Les Pluronics® L61 et P85 ont été inefficaces à augmenter l'accumulation de TPT dans les cellules MCF-7 TPT surexprimant la BCRP. Cependant les deux polymères ont augmenté l'accumulation de R123 dans les cellules surexprimant la BCRP et la MRP1. Finalement, une augmentation similaire d'accumulation de R123 a été occasionnellement observée dans les cellules MCF-7 parentales, MRP1 et BCRP négative. Cet effet noté ne semble pas être lié à la co-expression de la P-gp, puisqu'un manque d'activité des

inhibiteurs connus de la P-gp a été observé: vérapamil et cyclosporine A sur les cellules HL ADR, et GF120918 sur les cellules MCF-7 TPT. Également dans nos etudes, l'effet observé des polymères sur les cellules HL ADR ne peut pas être clairement attribué à la surexpression de la MRP1. Une observation importante a été faite au cours de l'étude portant sur la sélectivité du substrat pour les systèmes d'efflux P-gp, MRP1 et BCRP inhibés par les Pluronics® et par d'autres inhibiteurs. Les Pluronics® L61 et P85 ont eu un effet inhibiteur en augmentant l'accumulation de tous les substrats testés: R123, doxorubicine, MTX et TPT. Cependant, ces mêmes polymères ont seulement démontré l'effet sur l'accumulation de la R123 dans les cellules surexprimant MRP1 et BCRP. Les études de cytotoxicité ont démontré que l'effet de sensibilisation du Pluronic® L61 a été plus important pour les cellules MCF-7 ADR surexprimant la P-gp que pour les cellules HL 60 ADR exprimant la MRP1. Aucune sensibilisation n'a été observée sur les cellules MCF-7 TPT surexprimant la BCRP.

En conclusion, les Pluronics® sont très efficaces à inhiber l'efflux médié par la P-gp, moins efficace pour la MRP1 et démontre une efficacité limitée pour la BCRP. Évidement nos travaux représentent des études préliminaires portant sur l'effet des Pluronics® sur la résistance médiée par la BCRP. Dans le future, nous devrons prévoir des recherches plus détaillées en étendant le criblage des Pluronics® sur les cellules de type variées surexprimant la BCRP ou la MRP1. Nous devrons aussi inclure des investigations additionnelles portant sur l'influence des Pluronics® au niveau de la concentration intracellulaire d'ATP et au niveau de la viscosité membranaire cellulaire. De plus, pour des études plus détaillées sur les effets des Pluronics® et pour caractériser la contribution des transporteurs sur l'accumulation des substrats, des études à base d'expression et inhibition (expression par vecteur, siRNA) peuvent être envisagées. Ensuite ces études pourront étendre sur d'autres transporteurs.

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ABC	ATP-binding cassette
ABCB1	ATP-binding cassette, sub-family B, member 1 protein
ABCC1	ATP-binding cassette, sub-family C, member 1 protein
ABCG2	ATP-binding cassette, sub-family G, member 2 protein
ABCP	human placenta ABC protein
APO1	apoptosis inducing protein 1
ATP	Adenosine triphosphate
BBB	blood-brain barrier
BBMEC	bovine brain microvessel endothelial cells
Bcl-2	B-cell chronic lymphocytic leukemia /lymphoma 2 gene
BCRP	Breast cancer resistance protein
cDNA	complementary deoxyribonucleic acid
CMC	critical micelle concentration
CSA	Cyclosporin A
D-MEM	Dulbecco's Eagle medium
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
E2	17-β-Estradiol
EO	ethylene oxide
ER	endoplasmic reticulum
ERCC	excision repair cross-complementing rodent repair-deficiency
FL	fluorescein
FPR	fluorescent plate reader
GSH *S*	glutathione-sulfate
GSH	glutathione
GST	glutathione S-transferase
GS-X	glutathione S-conjugate export pump
HDL	high density lipoprotein
HL-60 ADR	adriamycin-resistant acute promyelocytic leukemia cell line

HL-60	acute promyelocytic leukemia cell line
HLB	hydrophilic–lipophilic balance
HPLC	High Performace Liquid Chromatography
IL	interleukin
IFN	interferon
MCF-7 ADR	adriamycin-resistant human breast cancer cell line
MCF-7 TPT	topotecan-resistant human breast cell line
MCF-7	human breast cancer cell line
MDR	multidrug resistance
MRP 1	multidrug resistance protein 1
MSD	membrane-spanning domains
MTX	Mitoxantrone
MXR	mitoxantrone resistance protein
NBD	nucleotide –binding domain
NBF	nucleotide-binding fold
NK	natural killer cells
p53	phosphoprotein *p53* gene
PBS	phosphate buffered saline
PEO	polyethylene oxide
P-gp	permeability-glycoprotein
PO	propylene oxide
PPO	polypropylene oxide
R123	Rhodamine 123
TPT	Topotecan
VLCFA	very long chain fatty acids
VP	Verapamil
XTT	2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H tetrazolium-5-carboxanilide sodium salt

Summary

Multidrug resistance (MDR) mediated by ATP-binding cassette (ABC) proteins, is one of the major reasons of therapeutic failure caused by malignant cancers and by many other diseases. To date, there are three clinically significant ABC transporters known to be associated with MDR: permeability protein (P-gp), multidrug resistant protein 1 (MRP1) and breast cancer resistant protein (BCRP). These transporters operate as "efflux pumps" removing xenobiotics from inside cells. P-gp is also responsible for limited absorption of a broad range of drugs from intestines to the blood stream. Modulation of the activity of ABC transporters may be essential for successful therapy of many diseases, and especially during chemotherapy. Several modulators of efflux systems have been previously tested. One type of modulator is the Pluronic® block copolymers (poloxamers), and they are of particular interest in our study as potential components of drug formulations. Pluronics® are composed of poly(ethylene oxide)(PEO) and poly(propylene oxide) (PPO) blocks, structurally arranged as $(PEO)_x(PPO)_y(PEO)_x$ (Schmolka, 1977). Previous *in vitro* and *in vivo* studies have demonstrated the ability of some Pluronics® to sensitize MDR cancer cells overexpressing P-gp (Alakhov et *al.*, 1996; Venne et *al.*, 1996, Batrakova et *al.*, 1996). In other studies in MRP1 and MRP2- transfected cells Madin Darby Canine Kidney (MDCKII), and in Dox resistant MRP1 overexpressing COR-L23\R lung cancer cells, it was demonstrated that Pluronic® P85 enhanced the accumulation of vinblastine and doxorubicin. However Pluronic® P85 had no effect in increasing Dox cytotoxicity in COR-L23/R resistant cells (Batrakova et *al.*, 2003).

The general objective of the present study was to examine *in vitro* the effects of Pluronic® block copolymers on the transport function of the three ABC proteins implicated in drug resistance: P-gp, BCRP and MRP1. More specifically, twenty-one co-polymers were tested to evaluate their critical micelle concentration (CMC), one of the basic surfactant properties. Further, the level of activity of Pluronics® in inducing the accumulation of rhodamine 123 (R123) was characterized in P-gp overexpressing breast cancer cell lines (MCF-7 ADR). Subsequently, Pluronics® L61 and P85 were chosen for next studies and were tested for their ability as potential inhibitors of P-gp, MRP1, and for the first time BCRP. For each transporter, absorption and cytotoxicity studies were conducted on doxorubicin resistant breast cancer cell lines (MCF-7 ADR) overexpressing P-gp, and the doxorubicin resistant leukemia cancer cell lines (HL-60 ADR)

overexpressing MRP1. Topotecan (TPT) resistant breast cancer cell subline (MCF-7 TPT) was derived by stepwise selection from parental MCF-7 cells exposed to TPT hydrochloride. The accumulation studies examined the influence of Pluronics® and other efflux inhibitors (cyclosporin A, verapamil, 17 ß-estradiol, GF120918) on intracellular accumulation of various probes (rhodamine 123, fluorescein, doxorubicin, mitoxantrone, topotecan). In addition, the inhibitory ability of Pluronics® was compared to that of other known efflux modulators listed above. The cytotoxicity tests determined the sensitization of drug resistant cells to cytotoxic drugs by Pluronic® L61. To do that, the cells were exposed to a drug-polymer treatment and the viability of cells was determined by a standard XTT test.

The results from the accumulation studies have shown that P-gp overexpressing MCF-7 ADR cells were the most responsive to the inhibitory effect of Pluronics®. Hydrophobic polymers like Pluronics® L61, L92 and P123 at their maximum effective concentration increased the accumulation of R123 in MCF-7 ADR cells more than the less hydrophobic polymer Pluronic® P85. Pluronics® showed only limited effectiveness in inhibiting BCRP and MRP1-mediated efflux. Pluronics® L61 and P85 failed to increase the accumulation of TPT in BCRP overexpressing MCF-7 TPT cells. However, both polymers enhanced the accumulation of R123 in BCRP and MRP1 overexpressing cells. In addition, a similar increase in R123 accumulation was occasionally observed in MRP1 and BCRP negative MCF-7 parental cells. The observed effect of polymers was found most likely to not relate to co-expression of P-gp, which was confirmed by the lack of any effect of well-known P-gp inhibitors: verapamil and cyclosporin A on HL 60 ADR cells and GF120918 on MCF-7 TPT cells. Additionally in our studies, the observed inhibitory effect of Pluronics® on HL 60 ADR cells could not be clearly attributed to MRP1 expression. A key observation made during this study was the substrate selectivity of P-gp, MRP1 and BCRP efflux systems inhibited by Pluronics®, and by other inhibitors. Pluronics® L61 and P85 showed an inhibitory effect by increasing the accumulation of all tested substrates: R123, Dox, MTX and TPT. However, the same polymers were only effective for the accumulation of R123 in MRP1 and BCRP overexpressing cells. The cytotoxicity studies showed that the sensitization effect of Pluronic® L61 was much greater for P-gp overexpressing MCF-7 ADR cells than for MRP1 overexpressing HL 60 ADR cells and

caused a decrease in Dox efflux. No sensitization was observed on BCRP overexpressing MCF-7 TPT cells. These findings further validate the inhibitory effect of Pluronics® on P-gp and MRP1 transporters.

In conclusion, according to our studies, Pluronics® are very effective in inhibiting P-gp, less effective in inhibiting MRP1 and exhibit limited effectiveness on BCRP-mediated drug efflux. Evidently our studies on BCRP-mediated efflux are still in the preliminary stage. In the future we should expand our research and carry out more detailed studies on various MRP1 and BCRP overexpressing cell types. Further screening of a wider range of Pluronics® should be performed on BCRP overexpressing cell types. This should include additional investigation of Pluronics® influence on intracellular ATP levels and on the viscosity of cell membranes. Also, to further study the effects of Pluronic® and characterize the contribution of transporters on substrate accumulation other studies using specific expression or inhibition (expression vector, siRNA) techniques should be performed. Those studies could also extend to other transporters. The data and the observations collected from this research are valuable building blocks that will complement our upcoming studies.

Introduction

Cancer is a cellular disorder characterized by abnormal and uncontrolled clonal expansion of cells, which often leads to invasion and destruction of healthy tissue. In the United States alone, since 1990 more than 17 million new cancer cases have been diagnosed. These numbers exclude noninvasive cancer and squamous cell skin cancers. Moreover, in the year 2006, about 564,830 patients were expected to die from the disease (*American Cancer Society*, 2006). The numbers are also serious in Canada with an estimated 153,100 new cases and 70,400 cancer deaths were expected in the year 2006 (Canadian Cancer Society /*National Cancer Institute of Canada*, 2006).

Three most common cancer treatments used solely or in combination are surgery, radiation and chemotherapy. Other treatments also comprise of biological and hormonal therapy such as the use of vaccines, interferons, monoclonal antibodies and blockage or modification of hormone production. Cancer treatment options depend on several factors such as the type and stage of cancer, patient's general health and the goal of the treatment; whether the therapy is to cure, to prevent from spreading or to relieve the pain caused by the disease. Often a successful therapy requires a combination of several treatments. Surgery is a local treatment and is ineffective to treat cancers that have already spread to lymph nodes, the blood and other parts of the body. Radiation is another local treatment, which is only capable of eradicating cancer cells at a specific site where the treatment is received. Radiation is administered by penetrating high-energy rays and damages the DNA of cancer cells in order to prevent them from growing and replicating. Cancer patients who do not respond to surgery or radiation and patients with haematological cancer are mainly treated with chemotherapy and immunotherapy (Gottesman et *al.*, 2002). Chemotherapy involves the use of cytotoxic drugs to eliminate cancer cells. Depending on the type and stage of cancer, sometimes drug therapy is the only effective treatment that a patient receives; often it is used as a complementary treatment to surgery, radiation and/or biological treatments (known as adjuvant chemotherapy). In some cases it may also be used to reduce the size of a tumor before surgery or radiotherapy (refereed to as neoadjuvant chemotherapy). Chemotherapeutic drugs may include: alkylating agents, antimetabolites, antitumor antibiotics, mitotic inhibitors and nitrosoureas. The treatment can be delivered orally in the form of a pill, by injection into a vein or into a muscle or topically. Various side effects can be observed as a result of the chemotherapeutic drugs,

which affect healthy cells as well as cancerous cells. Common side effects may include, fatigue, anemia, nausea, vomiting, hair loss, mouth sores and constipation (Mayo Clinic, 2005).

One major obstacle that often limits success of chemotherapy treatment is the ability of cancer cells to become resistant to different drugs leading to multidrug resistance (MDR) (Gottesman et *al.*, 2002). This phenomenon is characterized by cross-resistance of tumor cells to a broad range of structurally and mechanistically diverse drugs. There are several mechanisms leading to MDR, which will be briefly discussed in the following chapter of this thesis. However, active drug efflux mediated by the activity of several ATP-binding cassette (ABC) proteins that result in a decrease of cellular drug take is the sole mechanism of interest for our current study. Resistance can be "intrinsic" (inherent resistance) due to genetic/epigenetic alterations or "acquired" where upon cancer cells expose resistance to a large-spectrum of drugs before or after several rounds of chemotherapy (Johnstone et *al.*, 2000).

For years, cellular mechanisms of drug resistance have been intensively studied. One major cause of active drug efflux was found to be mediated by various mammalian membrane proteins belonging to the ABC superfamily (Schinkel and Jonker, 2003). Several of these proteins, operating like an "efflux pump", have significant impact on MDR cells and are present in variation in many tissues and cell types (higher expression in MDR cancer cell lines and tumors). They use free energy released during ATP hydrolysis to transport various molecules across cell membranes (Dean and Allikmets, 2001). The ubiquitous presence of ABC transporters has a beneficial effect on the proper function in biological systems. These proteins have numerous regulatory and protective functions. They play an important role in cellular defense mechanism against xenobiotics with broad substrate specificity. However, during drug administration the transport activity of the ABC transporters affects several pharmacological properties. These include low oral bioavailability, higher hepatobiliary, intestinal and urinary excretion of drugs, drug conjugates and metabolites. For example, during chemotherapy overexpression of certain ABC transporters often limit drug bioavailability and potency thereby causing treatment failure (Schinkel and Jonker, 2003). Three clinically significant ABC transporters associated with MDR are: permeability-glycoprotein (P-gp), multidrug resistant protein 1

(MRP1) and breast cancer resistant protein (BCRP). P-gp is the first identified and most widely studied drug efflux protein with resistance to a broad range of antineoplastics (Senior and Gadsby, 1997; Schinkel, 1997; Litman and Drule et *al*, 2000). MRP1 was identified in cell lines resistant to doxorubicin and was later found to confer resistance against numerous anticancer drugs. (Cole et *al.*, 1992; Cole et *al.*, 1994). Another ATP-dependent xenobiotic transporter, BCRP, was discovered in the human breast cancer subline and showed resistance to mitoxantrone, doxorubicin and daunorubicin (Doyle, Yang et *al.*, 1998).

Studies on multidrug resistance have shown that often there is an innate resistance towards most of the efficient drugs. Therefore, for years the focus was aimed towards finding novel treatments to circumvent drug resistance. One approach used to overcome MDR in cancer cells is administration of drugs with specific inhibitors of efflux pumps. For instance, cyclosporin A an inhibitor of P-gp and MRP1 was tested. However the immunosurpressive activity of this substance complicated its use in clinical oncology. Subsequently, PSC 833 (*Valspodar*) a cyclosporin A analogue without the immunosuppressive effect was introduced. One important difficulty related to the application of PSC 833 is its capacity to slow down cytochrome P450 3A4 (CYP3A4), a key drug-metabolizing enzyme in the body (Smith et *al.*, 1998). Later, another potent P-gp inhibitor, GF120918 (*Elacridar*) also known to be a non-specific BCRP inhibitor was tested in clinics (Hyafil et *al.*, 1993). Other MRP inhibitors like S-decylglutathione, benzbromarone and sulfinpyrazone have also been tested for their efficiencies. The poor specificity of cyclosporin A and the need for high dosage of other inhibitors such as benzbromarone and sulfinpyrazone suggests that better inhibitors of MRP are still needed (Hooijberg et *al.*, 1999; Evers et *al.*, 1996). BCRP inhibitors other than GF120918, fumitremorgin C, a tremorgenic mycotoxin produced by *Aspergillus fumigatus* is also known for its usefulness in BCRP-mediated drug efflux (de Bruin et *al.*, 1999; Rabindran et *al.*, 2000).

Other group of inhibitors of drug efflux transporters are some synthetic polymers. Surfactants, such as Cremophor, Tween 80 and especially some amphiphilic polymers of the Pluronic® family also known as poloxamers, have the ability to modulate multidrug resistance. Particularly, Pluronics® L61 and P85 are known to sensitize multidrug

resistance cells to various antineoplastics such as doxorubicin, epirubicin, vinblastine , mitomycin C and anthracycline antibiotics (Alakhov et *al.* 1996; Venne et *al.*, 1996; Batrakova et *al.* 2003). Pluronics® are amphiphilic block copolymers composed of poly(ethylene oxide)(PEO) and poly(propylene oxide) (PPO) blocks, structurally arranged as $(PEO)_x(PPO)_y(PEO)_x$ (Schmolka, 1977).

The mechanism of sensitization of MDR cancer cells by Pluronic® block copolymers has been investigated by Kabanov and Batrakova (Kabanov *et al.,* 2003; Batrakova *et al.*, 2003). Experimental studies have demonstrated that Pluronic® block copolymers sensitize several MDR cancer cells overexpressing P-gp, MRP1 and MRP2 transporters. Poloxamers are capable of fluidizing the cellular membrane, reaching the mitochondria and reducing ATP synthesis, resulting in energy depletion (Venne *et al.*, 1996; Kabanov *et al.,* 2003; Batrakova *et al.*, 2003). Cells expressing high levels of P-gp highly responded to Pluronic® P85 compared to cells expressing low levels of the same protein (Batrakova *et al.*, 2001).

In the following study of MDR efflux systems, the chemosensitizing activity of selected Pluronics® as potential inhibitors of P-gp, MRP1 and for the first time BCRP-mediated drug efflux was examined through a screening assay using fluorescence plate reader. The breast cancer cell lines, MCF-7 ADR (doxorubicin resistant) which overexpresses P-gp, MCF-7 TPT (topotecan resistant) which overexpresses BCRP and the leukemia cancer cell lines HL-60 ADR (doxorubicin resistant) which overexpresses MRP1 were selected for these studies. These cell lines conferred an MDR phenotype characterised by a reduction in the intracellular accumulation of cytotoxic agents. The uptake experiments were performed in presence of various substrates (rhodamine 123, fluorescein) including anti-cancer drugs (doxorubicin, mitoxantrone, topotecan) and in the presence of other known MDR inhibitors (cyclosporin A, verapamil, 17 ß-estradiol, GF120918). In addition, the surfactant properties of the selected Pluronics®, such as critical micelle concentration (CMC) were also evaluated.

The dissertation is divided into four chapters. The first chapter presents a detailed review of various mammalian membrane proteins belonging to the ATP-binding cassette (ABC) superfamily with a main focus on the three MDR efflux transporters; P-gp, MRP1 and BCRP. In addition, the properties of poloxamers of the Pluronic® family as MDR

modulators of active drug efflux are also addressed. The second chapter describes the materials and methods used to assess CMC, cytotoxicity and efflux studies. The third chapter presents research results obtained during the course of this study. The fourth chapter contain the discussion and a conclusion.

CHAPTER 1: Literature Review

1.1 Multidrug Resistance

In oncology, there are several causes for the ineffectiveness of anti-carcinogenic drug therapy. It can be related to difficulties in pharmacokinetics, such as the drug failing to reach the cells in sufficient dosage and in active form, to increased drug metabolism or excretion (Pluen et *al.*, 2001; Jain, 2001). Another reason for the chemotherapy failure is the resistance of cancer cells to a broad spectrum of structurally and functionally unrelated drugs referred to as "multidrug resistance" (MDR). This resistance can be "intrinsic"; when the cells are naturally irresponsive to the drug due to their indigenous properties, or "acquired"; when the treatment becomes non-effective to the malignancy after a relapse. For instance, multidrug resistance can be acquired in cancer cells through genetic alterations during the course of a treatment and the cells which becomes resistant proliferate if they posses a selective advantage (Kohichiroh et *al.*,. 2004).

Multidrug resistance is mediated by various mechanisms. The mechanisms which are implicated and clinically significant are: 1) modification of genes and proteins involved in managing apoptotic pathways (such as mutation of the *p53* gene and overexpression of Bcl-2 protein); 2) cellular drug detoxification by enzymes of the glutathione system; 3) activation of damaged cellular repair and alteration of drug targets (alteration of DNA repair and mutation of the topoisomerase II gene); 4) active drug efflux induced by transmembrane proteins (Figure 1.1), (O'Connor et *al.*, 1997; Stavrovskaya, 2000; Borst and Elfrenik, 2002).

1.1.1 Modifications of genes and proteins involved in managing apoptotic pathways

The tumor suppressor gene *p53* plays a significant role in regulating apoptosis and is an important element of a cell's response to injury. Specific mutations in *p53* can impair cells ability to repair its DNA and it also prevents cell cycle arrest or apoptosis in response to DNA damage. Gene *p53* also plays an essential role in MDR caused by gene modification. Thus alterations of p53 function can modify sensitivity of cells to drugs and induce resistance (Chumakov 2000; Kopnin, 2000).

Pten, a tumor suppressor gene, negatively regulates the phosphatidylinositol 3'-kinase protein kinase B (PKB)/Akt (thymoma viral proto-oncogene) signalling pathway. PKB/Akt is a regulator of cell survival. Inactivation of Pten can decrease cell sensitivity to

chemotherapy, thus inducing drug resistance (Stambolic et *al.*,1998). Studies have also shown that overexpression of oncogene *Bcl-2*, an inhibitor of apoptotic cell death, induces resistance to different drugs and contributes to metastasis in certain cancers (Dive, 1997).

1.1.2 Cellular detoxification by enzymes of the glutathione system

The glutathione (GSH) system plays a significant role in cellular detoxification of many xenobiotic compounds. Glutathione, a non-protein thiol, is capable of conjugating with a drug, forming more hydrophilic entities, which are removed from the cells with the aid of transporter proteins such as GS-X (ATP-dependent glutathione S-conjugate export pump) and MRP. The isozymes of glutathione S-transferases (GST) are proficient catalyzers for the conjugation of glutathione with alkylating drugs and consequently increase the rate of drug detoxification, which can result in cellular drug resistance (Tew, 1994). Increased levels of GST*pi* isozyme were associated with resistance to alkylating agents like, nitrogen mustard, cyclophosphamide and melphalan (Tew, 1994). To regulate this resistance, inhibitors of GST-mediated drug resistance such as buthionine sulfoximine, and ethacrynic acid (a diuretic) have been tested. These inhibitors modulate drug resistance by decreasing intracellular levels of glutathione and preventing its interaction with alkylating agents. Ethacrynic acid showed inhibitory activity *in vitro* but the lack of isoenzyme specificity and undesirable side effects restricted its clinical use (Tew 1994; Tew and Gate 2001).

1.1.3 Activation of damaged cellular repair and alteration of drug targets

Several anticancer drugs target topoisomerase II, an enzyme that catalyzes the transient breaking and rejoining of DNA. These drugs inhibit topoismorease II and operate like a poison by increasing the level of enzyme-mediated cleavage in the genetic material of the treated cell and eventually trigger cell death (Fortune and Osheroff, 2000). A mutation of the gene encoding topoisomerase II as well as defects in topoisomerase phosphorylation are also believed to cause some cases of this type of drug resistance (Pommier, et *al.*, 1994; Takano et *al.*, 1991; DeVore et *al.*, 1992).

Alternatively, resistance can occur as a result of activation of proteins recognizing and repairing drug-induced DNA damage. For instance during a study involving cultured

cells resistant to a platinum derivative drug, DNA repair proteins ERCC1, ERCC2, ERCC3/XPB were discovered (Chu, 1994; Stavrovskaya, 2000).

1.1.4 Active drug efflux

Current treatment of cancer use combination of anticancer drugs that affect multiple targets at once. Consequently, the occurrence of MDR is a major problem for combination chemotherapy involving several antineoplastics. Active drug efflux studied both *in vitro* and *in vivo* is one of the most common mechanisms of MDR involving the expression of the membrane proteins belonging to the ATP-binding cassette superfamily. In this study, active drug efflux will be the only mechanism of interest.

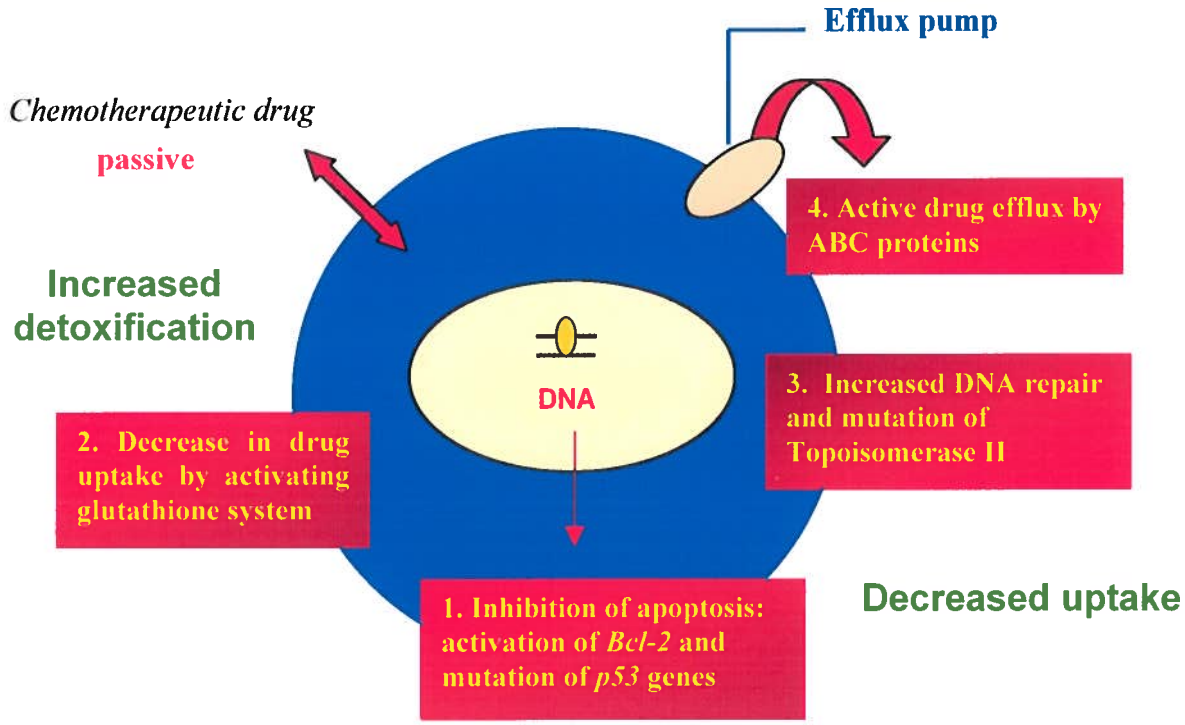


Figure 1.1 Various multidrug resistance mechanisms.
In eukaryotic systems, administration of antineoplastics can lead to activation of numerous multidrug resistance mechanisms. These systems have been identified as: 1) inhibition of apoptosis by activation of *Bcl2* and mutation of *p53* genes which are involved in cell apoptosis 2) decrease in drug uptake by activating enzymes of the glutathione system 3) increased DNA repair which is due to changes in the quantities of proteins recognizing and repairing DNA injury 4) active drug efflux initiated by proteins of the ATP binding cassette (ABC) transport systems.
Adapted from (Gottesman et *al.*, 2002).

1.2 The ATP-binding cassette (ABC) superfamily

The ATP-binding casette (ABC) superfamily (also known as traffic ATPases) is the largest family of transmembrane proteins present in both eukaryotes and prokaryotes systems. These functionally diverse proteins are involved in various functions and work by utilizing the energy of ATP to transport a variety of substrates across extra- and intra-cellular membranes (Ames and Lecar, 1992; Childs and Ling, 1994). Basic structural features that defines these proteins as ABC transporters is their sequence homology and organisation of their ATP-binding domains, also referred to as nucleotide binding domain (NBD) or nucleotide-binding folds (NBFs) (Dean , 2002). Overall in ABC transporters, the sequence of NBFs always contains the ATP-binding motifs Walker A and Walker B distanced by ~90-120 amino acids and ends with a distinctive LSGGQ motif also known as C motif or "signature motif" positioned upstream of the Walker B site (Hyde et *al.*, 1990). A functionally active ABC transporter generally is comprised of two NBFs and two transmembrane (TM) domains (Figure 1.2). The TM domains enclose 6-11 membrane-spanning regions made of α-helices and set the specificity for the type of molecule to be transported. NBFs are located in cytoplasm and provide the energy derived from ATP hydrolysis to translocate the molecules across the membrane. In bacteria, ABC transporters are involved in the import of nutrients (vitamins, metal ions, lipids, sugars) into the cell, whereas in eucaryotic system they export mainly hydrophobic molecules within the cell compartments or out of cell (Dean , 2002). Functionally active ABC transporters are identified as full transporters (made of two symmetric halves with two TM and two NBFs domains aligned in one polypeptide), or as half transporters (made of two separate polypeptides each comprising one TM and one NBF fused by homo- or heterodimerization) (Klein et *al.*, 1999). Young and Holland also stressed within their research the distinction between the terms ABC proteins and ABC transporters by stating that ABC proteins are comprised of ATP-binding cassette and NBF however, ABC transporters other than including the latter also contain additional transhydrophobic membrane domains with translocation function. They also suggested the omission of the term "substrates" for any compound transported by ABC transporters given that they are not enzymatically modified during translocation and instead, they proposed the term "allocrite" (Paulsen et *al.*, 1996; Young and Holland, 1999). Nonetheless, scientific papers

regarding this subject are continuously using the term "substrate", and for that reason throughout this thesis the same term will be employed.

a)

b)

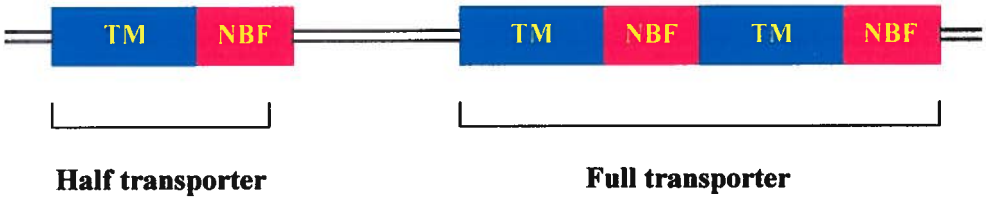


Figure 1.2 Topology of ABC transporter protein and its gene structure
a) The nucleotide binding folds (NBF) of all ATP-binding cassette (ABC) gene contains the ATP-binding motifs Walker A and Walker B distanced by ~90-120 amino acids and ends with a distinctive LSGGQ motif also known as C motif.
b) ABC transporter proteins are functionally active when identified as full transporters {made of two symmetric halves with two transmembrane (TM) domains and two NBFs for ATP-binding aligned in one polypeptide}, or as half transporters {made of two separate polypeptides each comprising one TM and one NBF merged by homo- or heterodimerization}
Adapted from Micheal Dean's book: "The Human ATP-Binding Casette (ABC) Transporter Superfamily", Nov.2002.

1.2 Overview of human ABC transporter gene subfamilies

In humans, ABC transporter genes are divided into seven subfamilies: *ABCA*, *ABCB*, *ABCC*, *ABCD*, *ABCE*, *ABCF* and *ABCG*. Each subfamily comprises various members with diverse regulatory functions. At present, 48 human ABC transporters have been identified (Figure 1.3). So far, the *ABCG3* gene has not been found in humans however, high levels of its expression in mice were detected suggesting that *ABCG3* might also be expressed in humans. A summary describing 48 human ABC transporters and their basic features are shown on (Table 1.3). A concise overview of these genes is also summarized by Michael Müller, University of Wageningen, The Netherlands (http://www.nutrigene.4t.com/humanabc.htm).

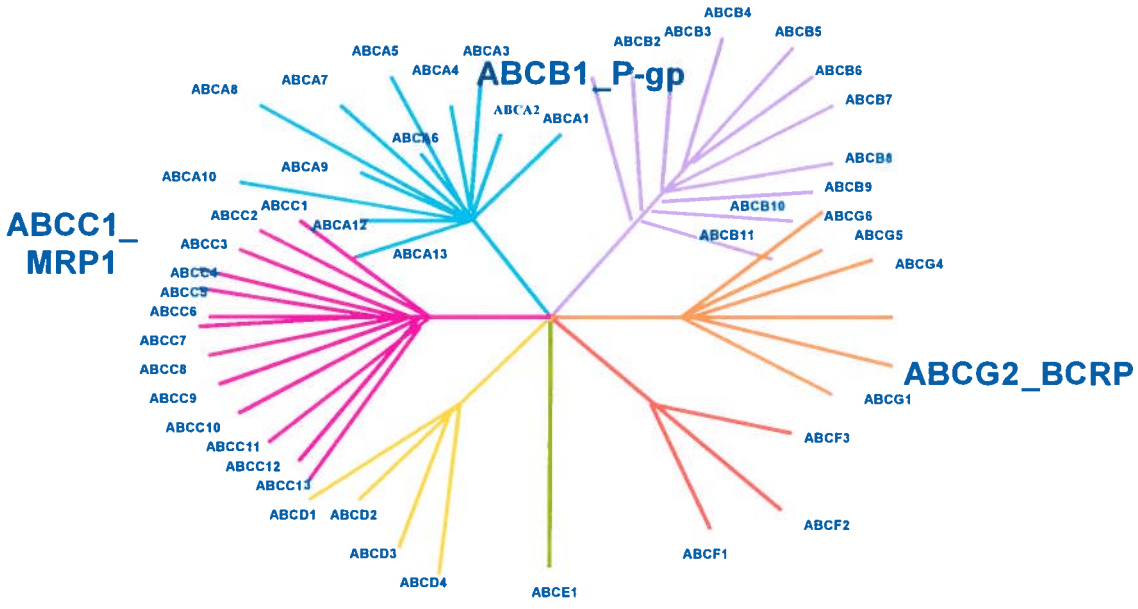


Figure 1.3 Human ABC transporter subfamily tree.
Human ABC transporters are grouped into seven subfamilies: ABCA, ABCB, ABCC, ABCD, ABCE, ABCF and ABCG. P-gp, MRP1 and BCRP1 have demonstrated a well-defined role in the transport of clinically relevant drugs.
Adapted from (Doyle and Ross, 2003).

Table 1.3 Human ABC transporters, characteristics, function and associated diseases

Family	Member	Alias	Expression	Function	Associated disease by ABC genes
ABCA	ABCA1	ABC1	Ubiquitous	Cholesterol and phospholipids efflux onto HDL	Tangier, FHDLD
	ABCA2	ABC2	Brain, lung	Lipid transport, drug resistance	
	ABCA3	ABC3, ABCC	Lung	Regulating lipid transport (for lamellar bodies)	
	ABCA4	ABCR	Retina	N-retinylidene-phosphatidylethanolamine efflux	Stargardt , FFM, RP,CRD
	ABCA5		Muscle, heart, testes		
	ABCA6		Liver	Possible role in macrophage lipid homeostasis	
	ABCA7		Spleen, thymus	Possible role in lipid transport	
	ABCA8		Ovary		
	ABCA9		Heart	Cholesterol regulation in macrophage	
	ABCA10		Heart, muscle		
	ABCA12		Stomach		
	ABCA13		Trachea, testis, bone marrow		
ABCB	ABCB1	MDR1, P-gp	Kidney, brain	Multidrug resistance	
	ABCB2	TAP1	Ubiquitous, ER	Peptide transport into endoplasmic reticulum	TAP1and 2: Ankylosing spondylitis, IDDM, celiac disease
	ABCB3	TAP2	Ubiquitous, ER	Peptide transport into endoplasmic reticulum	
	ABCB4	MDR3, PGP3	Liver Ubiquitous	Phosphatidylcholine transport	PFIC3
	ABCB5		Mitochondria		
	ABCB6	MTABC3	Mitochondria	Iron transport	LNMS
	ABCB7	ABC7	Mitochondria	Heme transport	XLSA/A
	ABCB8	MABC1	Heart, brain	Heme trasnport	
	ABCB9		Mitochondria		
	ABCB10	MTABC2	Liver		
	ABCB11	SPGP, BSEP		Bile salt transport	PFIC2
ABCC	ABCC1	MRP1	Ubiquitous	Multidrug resistance	
	ABCC2	MRP2, cMOAT	Liver, intestine	Organic anion efflux	Dubin-Johnson syndrome
	ABCC3	MRP3	Intestine, kidney, liver	Drug resistance	
	ABCC4	MRP4	Ubiquitous	Nucleoside transport	
	ABCC5	MRP5	Ubiquitous	Nucleoside transport	
	ABCC6	MRP6	Kidney, Liver		Pseudoxanthoma elasticum
	ABCC7	CFTR	Lung, intestine, cholangiocytes	Chloride ion transport	Cystic Fibrosis, CBAVD
	ABCC8	SUR1	Pancreas	Sulfonylurea receptor	FPHHI, NIDDM2
	ABCC9	SUR2	Heart, muscle		
	ABCC10	MRP7	Low in all tissues		
	ABCC11	MRP8	Low in all tissues		
	ABCC12	MRP9	Low in all tissues		
ABCD	ABCD1	ALD	Peroxisomes	VLCFA transport regulation	ALD
	ABCD2	ALD1, ALDR	Peroxisomes		
	ABCD3	PMP70, PXMP1	Peroxisomes		
	ABCD4	PMP69, P70R	Peroxisomes		
ABCE	ABCE1	OABP, RNS41	Ovary, testes , spleen	Oligoadenylate-binding protein	
ABCF	ABCF1	ABC50	Ubiquitous		
	ABCF2		Ubiquitous		
	ABCF3		Ubiquitous		
ABCG	ABCG1	ABC8, White	Ubiquitous	Cholesterol transport	
	ABCG2	ABCP, BCRP, MXR,	Placenta, intestine	Multidrug resistance	
	ABCG4	White 2	Liver		
	ABCG5	Sterolin 1	Liver, intestine	Sterol transport	Sitosterolemia
	ABCG8	Sterolin 2	Liver, intestine	Sterol transport	Sitosterolemia

Adapted from a review by (Glavinas et *al.*, 2004). HDL: high density lipoprotein; FHDLD: familial hypoapoproteinemia; FFM : fundus flavimaculatis; RP : retinitis pigmentosum; CRD : cone-rod dystrophy; IDDM : insulin-dependent diabetes mellitus;PFIC : progressive familial intrahepatic cholestasis; LNMS: lethal neonatal metabolic syndrome; XLSA/A: X-linked siderblastic anemia with ataxia; PFIC: progressive familial intrahepatic cholestases; CBAVD: congenital bilateral absence of the vas deferens; FPHHI: familial persistent hyperinsulinemic hypoglycemia of infancy; NIDDM: non-insulin dependent diabetes mellitus; ALD: adrenoleukodystrophy.

ABC transporters are also referred to by other names that are derived from their functionality or the diseases with which that they are associated. ABCA, ABCB, ABCC and ABCF are expressed in many tissues. ABCG is mainly located in the brain, placenta, liver, intestines and lungs. On the other hand, ABCD is solely expressed within peroxisomes. ABC transporters all have special functions as cellular regulators or protectors from invasive toxic substances that might alter the cells. For example ABCA1 functions as a key regulator of in high-density lipoprotein (HDL) and cellular cholesterol homeostasis. Some of these ABC genes are also associated with various diseases as listed in Table 1.3.

To date, only P-gp, MRP1 and BCRP are known to be implicated in MDR. Especially in clinical studies, these three proteins are of particular importance because they affect the success of chemotherapy treatment. Their presence is associated with increased resistance to a variety of anticancer drugs. Many studies have been undertaken to circumvent this resistance therefore, we will limit our interest in this study to the P-gp, MRP1 and BCRP efflux systems.

1.4 P-glycoprotein, MDR1

1.4.1 General properties and mechanism of P-glycoprotein, MDR1

Permeability-glycoprotein, (also known as P-glycoprotein, P-gp, ABCB1, MDR1) is a 170-kDa phosphorylated and glycosylated transmembrane protein belonging to the ABC superfamily and consisting of 1280 amino acids. Being the first identified transporter that was implicated in MDR, P-gp was initially discovered by Juliano and Ling in Chinese hamster ovary cells resistant to colchicines (Juliano and Ling, 1976). From there on, various isoforms of this protein were detected in humans, mice and rats with high gene homology between and within species. In humans, P-gp is encoded by the *MDR1* gene and in rodents it is encoded by three genes: *mdr1a* (also called *mdr3*), *mdr1b* (also known as *mdr1*) and *mdr2* (Ueda et *al.*, 1987; Hsu et *al.*, 1989). Only the *MDR1*gene in humans and *mdr1b*, *mdr1a* genes in rodent appear to confer drug resistance. P-gp is a full-transporter and consists of two homologous halves each comprising 610 amino acids joined together by a cytoplasmatic linker of about 60 amino acids (Chen et *al.*, 1986). Each half contains a N-terminal hydrophobic domain with six putative TM segments for substrate binding, followed by a hydrophilic domain containing one NBF for ATP binding (Figure 1.4). As a result of combining these halves, P-gp operates as a fully active single transporter (Loo and Clarke, 1994). P-gp undergoes significant conformational changes during drug interaction and the contact between the NBFs and the TM domains are essential for the proper function of drug transfer (Rosenberg et *al.*, 2003).

An increased expression of P-gp on the cell surface is a major cause of intrinsic and acquired resistance to drugs (Ueda et *al.*, 1987; Gottesman and Pastan, 1993). To date, the exact mechanism by which P-gp is able to transport a vast range of substrates is still under some matter of debate. However, all models agree on P-gp utilizing the energy derived from ATP hydrolysis to transport various substrates. Two ATP molecules are hydrolysed for the transport of one substrate molecule (Gottesman and Pastan, 1993). One of the ATP molecules is implicated in the conformational change within the TM domains, translocating substrates out of the cell and the second one is involved in restoring P-gp back to its original state for the next catalytic cycle (Sauna and Ambudkar, 2000).

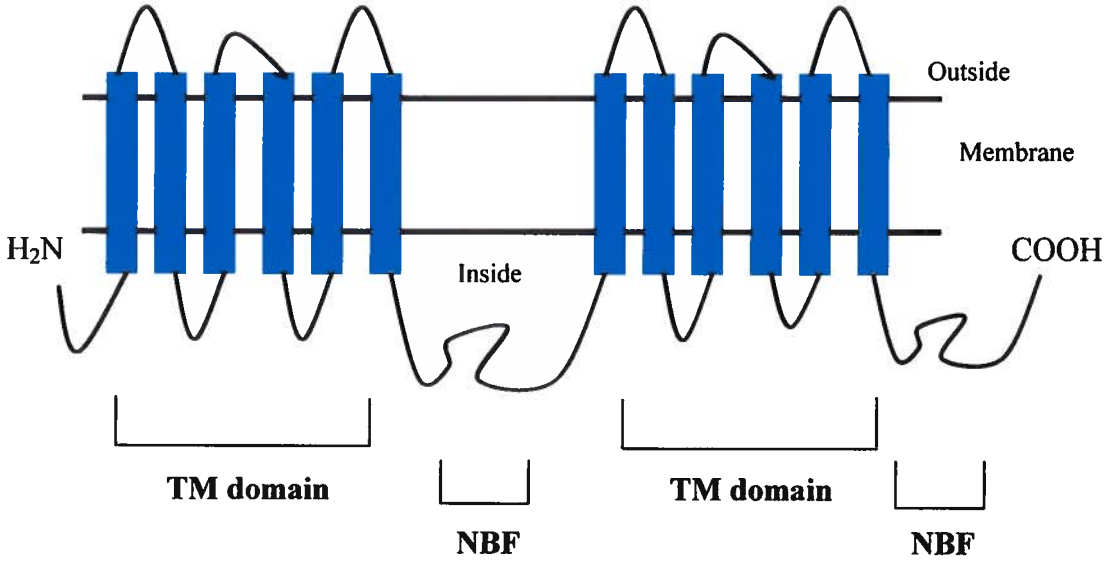


Figure 1.4 Membrane topology of P-glycoprotein (P-gp)
P-gp is comprised of 12 transmembrane (TM) domains, consisting of two analogous halves joined together by a cytoplasmatic linker. Each half contains a N-terminal hydrophobic domain with six putative α-helical TM segments for substrate binding followed by a highly conservative nucleotide-binding fold (NBF) for ATP binding.
Adapted from (Leonard et *al.*, 2003).

Throughout the years, several models have been proposed. A concise overview of these models has been published by Shannon Mala Bard (Bard, 2000). Early findings suggested that P-gp is functioning both as an ATP conducting channel and swelling-activated chloride-selective channel (Abraham et *al.*, 1993; Valverde et *al.*, 1992). For a long time, P-gp involvement in the regulation of swelling-activated chloride channels has been a subject of some debate. However, several studies strongly suggest that the P-glycoprotein regulates the activity of chloride channels in some cell types (Higgins, 1995; Marin et *al.*, 2005). The classical pump model for P-gp, "*the pore model*", introduced the transporter to develop an aqueous pore, which undergoes conformational rearrangement in order to transport hydrophilic substrates from cytosolic compartment to outside the cell through a protein channel (Borst and Schinkel 1997). This proposition required several modifications considering that P-gp has a broad spectrum of hydrophobic substrates and the binding between the transporter and the substrate occurs in the lipid bilayer of the membrane (Sharom, 1997). Subsequently, three other models were proposed to elucidate the mechanism of action employed by this transporter. One of the models suggested that P-gp might increase drug efflux and reduce the intracellular entry of a drug by acting as a "*hydrophobic vacuum cleaner*". The model proposed that P-gp (having non-selective binding sites) directly bind to drugs from the inner or outer leaflet of the phospholipid bilayer hence, transporting them from the plasma membrane before reaching the cytosol and extruding them into the extracellular medium (Higgins and Gottesman, 1992; Gottesman and Pastan 1993). However, this model was still not completely satisfactory because P-gp was shown to specifically bind substrates having diverse specificities (Liu and Sharom, 1996). The next model introduced a "*flippase*" function. Flippases are membrane enzymes that switch leaflets into phospholipids. Phospholipids are laterally mobile however the movement between one leaflet of the lipid bilayer to the opposing leaflet is poor because of the high-energy barrier to be overcome in translocating its polar headgroup through the hydrophobic interior of the membrane (Menon, 1995). Flippases, accelerate the rate of this process and are driven by ATP hydrolysis. The "*flippase model*" was introduced to describe the ability of P-gp to flip drugs from the inner to the outer leaflet of the lipid bilayer against a concentration gradient and then passively diffuse them into the external medium (Figure 1.4.1) (Higgins and Gottesman, 1992). Eytan and Kuchel

(1999), proposed another model which combined the rudiments of the last two models. Later, this model was further elaborated upon by Eytan (2005). They first described the binding of positively charged hydrophobic P-gp substrates from the extracellular space into the negatively charged outer leaflet of the lipid bilayer. This results in equilibrium between the high concentration cellular bound drug pool present in the outer leaflet of the membrane compared to the low concentration drug pool in the medium. Drugs cross the inner and outer lipid bilayer by slow flip-flop events and do not diffusing down a concentration gradient. Drugs in the inner leaflet reach a practical equilibrium with the drug pool in the cytoplasm, which are bound by molecular sinks such as DNA or cytoskeleton elements. Substrates in the inner leaflet bind to P-gp and are actively flipped out (pumped) to the extracellular phase (Eytan and Kuchel, 1999; Eytan 2005).

In humans, P-gp is expressed in the epithelia of the liver, the renal proximal tubules, the epithelial apical membrane of the intestine, ovary, testis, luminal membrane of endothelial cells in brain and in the apical membrane of the syncytial trophoblasts of the placenta (Asperen et *al.*, 1998; Thiebaut *et al.*, 1987; Asperen et *al.*, 1997). Besides being expressed in various non-malignant tissues, P-gp is also expressed in many MDR cancer cells (Fojo et *al.*, 1987). In several studies, at the time of diagnosis, intermediate levels of P-gp expression have been observed in some neuroblastomas and soft tissue carcinomas, CD34-positive acute myeloid leukemias and chronic myeloid leukemias (Bourhis et *al.*, 1989; Drenou et *al.*, 1993; te Boekhorst et *al.*, 1993). Beside these malignancies, tumors of the stomach, esophagus, ovary, lung and breast showed low levels of P-gp (Moscow et *al.*, 1989; Arao et *al.*, 1994; Lai et *al.*, 1989; Merkel et *al.*, 1989). After chemotherapy treatment and with the emergence of acquired drug resistance, high levels of P-gp were observed in breast tumors, acute myeloid leukemias, lymphomas and myelomas (Schneider et *al.*, 1989; Marie et *al.*, 1991; Cheng et *al.*, 1993; Petrini et *al.*, 1995).

P-gp expression is regulated by various factors, such as interaction with xenobiotics, hormones, heat shock proteins, oncogene transfection, genotoxic stress, cell differentiation or proliferation, inflammatory mediators and cytokines (Fardel et *al.*, 1996; Petrini and Galimberti, 1997; Sukhai and Piquette-Miller, 2000).

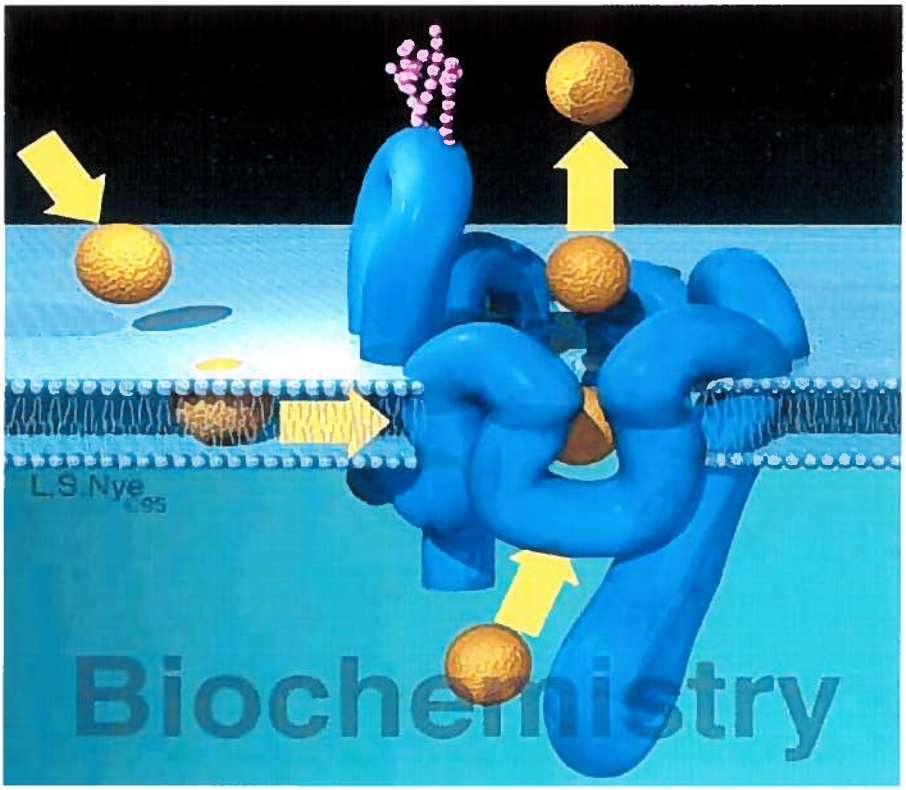


Figure 1.4.1 Mechanism of action of P-glycoprotein (P-gp)
This figure represents one of the proposed mechanisms for P-glycoprotein. This is not a real simulation but rather an artistic presentation of the protein in action.
The latest proposed mechanism by Eytan and Kuchel suggests that the cationic hydrophobic substrates from the extracellular phase of the lipid bilayer bind to the negatively charged outer leaflet. This results in equilibrium between the high concentration cellular bound drug pool present in the outer leaflet of the membrane compared to the low concentration drug pool in the medium. Then substrates slowly cross the lipid membrane by a flip-flop "*flippase*" mechanism. Inside the inner leaflet substrates bind to P-gp and are actively pumped (flipped) out of the cell (Eytan and Kuchel 1999; Eytan, 2005).
Image created by Linda S. Nye, "*P-glycoprotein Responsible for Multidrug Resistance in Chemotherapy*" (included in the thesis with the permission of the artist)
http://www.medartist.com/nye.html#Contact

1.4.2 Physiological roles of P-glycoprotein

P-gp plays an important role in protecting various parts of the body against the absorption and distribution of toxic compounds and metabolites. P-gp functions as a xenotoxin barrier for the entry of substrates and as well as an efflux pump to expel substrates mainly from the brain, testis, nerves and placenta. The blood-brain barrier (BBB) is composed of endothelial cells that are closely linked to each other by tight junctions in the capillaries of the brain (Engelhardt, 1997). BBB excludes almost all molecules from the brain, with the exception of small and lipophilic molecules (Rubin and Staddon, 1999). P-gp is present in high amounts on the apical surfaces of endothelial cells and is involved in protecting the brain from exposure to various xenotoxins including small and hydrophilic molecules (Thiebaut et *al.*, 1989; Cordon-Cardo et *al.*, 1989).

P-gp also possesses barrier function against xenotoxins in blood-brain, blood-testis, blood-nerve and in the placental syncytiotrophoblasts (Schinkel et *al.*, 1995; Schinkel et *al.*, 1996; Choo et *al.*, 2000; Saito et *al.*, 2001; Lankas et *al.*, 1998). In addition, P-gp is suggested to play a role in phospholipid translocation and in intracellular cholesterol esterification (van Helvoort et *al.*, 1996; Luker et *al.*, 1999; Garrigues et *al.*, 2002).

Experimental evidence indicates that P-gp might be involved in the transport of cytokines, particularly IL-1ß, IL2, IL4 and IFN-γ and it may play a role in increasing effector cell function (Young and Krasney, 1992; Drach et *al.*, 1996). During several investigations relatively high expression of P-gp was observed on NK and $CD8^+$ T immune cells capable of inducing cell death via the Fas/Fas ligand pathway or by release of cytotoxic T lymphocytes granules components perforin and granzymes into the target cell (Smyth and Trapani, 1995). Several studies have shown a reduction on NK and $CD8^+$ T-cell cytolytic activity when P-gp efflux function was decreased (using P-gp inhibitors) or when the expression of P-gp was low (using anti-sense oligonucleotides) (Gupta, 1992; Chong, 1993).

In addition, P-gp may participate in programmed cell death mediated by some chemotherapeutic drugs, ultraviolet irradiation, serum starvation, tumor necrosis factor receptor and ligation of cell receptors Fas (Robinson et *al.*, 1997; Johnstone et *al.*, 1999).

A study by Robinson et *al.* demonstrated that P-gp expressing MDR1 induced resistance to apoptosis by serum starvation, which could be reversed by addition of verapamil, a competitive inhibitor of P-gp (Robinson et *al.*, 1997). Further, a family of cysteine proteases known as caspases is known to mediate the activation of cell-death. An investigation has shown that upon Fas ligation, P-gp can inhibit caspase-dependent cell death. Moreover this inhibition was reversible with the addition of verapamil or anti-P-gp monoclonal antibodies (Smyth et *al.*, 1998; Johnstone et *al.*, 1999).

P-gp is also highly expressed in the bilary canalicular membrane of hepatocytes and in the apical surface of jejunum and colon epithelium (Thiebaut et *al.*, 1987). Therefore, P-gp plays a major role in limiting the oral bioavailability of drugs. Studies in knockout animals showed that P-gp is actively involved in the extrusion of substrate drugs from the blood into the bile and from the intestinal epithelium into the intestinal lumen (Mayer et *al.*, 1996; Sparreboom et *al.*, 1997). In addition, in kidneys, P-gp is highly expressed in the apical membrane of proximal tubules, however the exact function of P-gp in kidneys is still unclear (Thiebaut et *al.*, 1987).

Further, recent discoveries have suggested the involvement of P-gp in the efflux of ß-amyloid, where by, the increase of this protein in the brain contributes to the aetiology of Alzheimer's disease (Lam et *al.*, 2001). Expression of P-gp in the adrenal glands also suggests a role in the transport of steroids like dexamethasone, cortisol and aldosterone (Ueda et *al.*, 1992).

1.4.3 Substrates and inhibitors of P-glycoprotein

P-gp interacts with a variety of compounds that are structurally and functionally diverse. To date, the common features of P-gp substrates are still not precisely known, and structural predictions that were made over the years are still far from being effective. P-gp exhibits broad affinity to various molecules and actively plays a role as a "*gate keeper*", preventing the entry or absorption of toxic materials into the vital organs. Some of the common features of P-gp substrates have been identified as low molecular weight, anions, cations, zwitterions, neutral compounds, aromatic componds, peptides and non-peptides

(Pearce et *al.*, 1989). P-gp substrates conferring MDR include anthracyclines, vinca alkaloids, epipodophyllotoxins, taxanes, cytotoxic agents, linear and cyclic peptides, steroids, color probes and some other compounds (Sharom, 1997). A more detailed list of P-gp substrates is shown in Table 1.4.3.

The presence of P-gp in various parts of the body, especially in many tumor cells, has been observed to interfere with the delivery of drugs to target tissues. Over the years, as a solution to increase the bioavailability of drugs and prevent drug interference with P-gp, several potent and relatively non-toxic P-gp modulators also known as "inhibitors" or "blockers" were developed. Ultimately the effectiveness of some of these potential inhibitors is still in study before reaching a final conclusion on their usefulness. Generally, modulation of P-gp or other efflux transporters can be achieved: (a) by blocking the drug binding site either competitively, non-competitively or allosterically; (b) by inhibiting ATP-hydrolysis; or (c) by modifying cell membrane lipid composition. Most drugs however, inhibit P-gp function by blocking drug-binding sites where substrates and inhibitors are handled (Varma et *al.*, 2003). For years, a major question that has been raised concerning P-gp transport mechanism is: "why aren't P-gp inhibitors transported in the same way as the P-gp substrates?" Some P-gp inhibitors turned out to be themselves transported by P-gp, which are referred as "competitive inhibitors". However, the efflux rates of these inhibitors compared to other substrates are slower. Eytan *et al.* proposed a reasonable explanation to this mechanism by suggesting that inhibitors transported by P-gp could rapidly return to the inner leaflet of the bilayer for further transport. In such momentum that these repeated cycles, create a larger gap between the expulsion rate of the substrate and the inhibitor. The inhibitors returning to the inner leaflet prevent substrates from being transported out and are mainly dependent on the hydrophobicity of the compound (Eytan et *al.*, 1996).

Table 1.4.3 List of P-gp substrates

Anthracyclines	**References**
Doxorubicin	Mulder et *al.*, 1995
Daunorubicin	Kaye and Merry, 1985
Epirubicin	Mulder et *al.*, 1995
Idarubicin	Maia et *al.*, 1998
Vinca alkaloids	
Vinblastine	Horio et *al.*, 1988
Vincristine	Fojo et *al.*, 1985
Vinorelbine	Fojo et *al.*, 1985
Taxanes	
Paclitaxel	Gottesman et Pastan 1993
Docetaxel	Gottesman et Pastan 1993
Epipodophyllotoxins	
Etoposide	Lum et *al.*, 1993
Teniposide	Lum et *al.*, 1993
Cytotoxic agents	
Colchicine	Doige and Sharom, 1992
Actinomycin D	Horio et *al.*, 1989
Puromycin	Kondratov et *al.*, 2001
Mitoxantrone	Taylor et *al.*, 1991
Bisantrene	Zhang et *al.*, 1994
Topotecan	Hoki et *al.*, 1997
Immunosurpressive agents	
Methotrexate	Gifford et *al.*, 1998
HIV Protease Inhibitors	
Nelfinavir	Hennessy et *al.*, 2004
Indinavir	Lee et *al.*, 1998
Ritonavir	Lee et *al.*, 1998
Amprenavir	Polli et *al.*, 1999
Sequinavir	Lee et *al.*, 1998
Antidiarrheal agent	
Loperamide	Wandel et *al.*, 2002
Domperidone	Dan et *al.*, 2002
Ondansetron	Schinkel et *al.*, 1996
Antibiotics	
Erythromycin	Kim et *al.*, 1999

Cardiac drugs	**References**
Digoxin	Tanigawara et *al.*, 1992
Lovastatin	Kim et *al.*, 1999
Linear and cyclic peptides	Sharom et *al.*, 1998
Leupeptin	
Gramicidin D	
Pepstatin A	
Nonactin	
NAc-Leu-Leu-norLeu-al	
NAc-Leu-Leu-Met-al	
Yeast a-factor	
Steroids	
Dexamethasone	Nakayama et *al*, 1999
Cortisol	Farrel et *al.*, 2002
Corticosterone	Wolf and Horwitz, 1992
Aldosterone	Bello et *al.*, 2000
Hydrocortisone	Nakayama et *al*, 1999
Analgesics	
Morphine (poor substrate)	Wandel et *al.*, 2002
Meperidine	Thompson et *al.*, 1999
Diagnostic dyes	Kessel et *al.*, 1991
Rhodamine 123	Shapiro and Ling 1997
Hoechst 33342	Agrawal et *al.*, 2003
^{99m}Tc-SESTAMIBI	
Antiparsitics	Griffin et *al.*, 2005
Ivermectin	

Based on their affinity and specificity, P-gp inhibitors are divided into three generations (Table 1.4.3.1 modified from Varma et *al.*, 2003). First-generation inhibitors include calcium channel blockers like verapamil, immunosuppressant such as cyclosporin A, antiestrogens like toremifena and tamoxifen, and anti-hypersensitives like quinidine and reserpine. The therapeutic application of these inhibitors is restricted, due to their specific pharmacological activities and toxicity when used in high dosage to obtain an inhibitory effect. For example verapamil is a competitive P-gp inhibitor, and it's elevated concentration in plasma is required to achieve an inhibitory effect that can be seriously toxic. Consequently, after years of research by various scientific groups, second and third generation modulators were introduced. Second-generation inhibitors like PSC 833 (*valspodar*, a non-immunosurpressive analogue of cyclosporin A), D-isomer of verapamil, dexverapamil, VX-710 (*biricodar*), MS-209 and GF120918 (*elacridar*, also called GG918) were developed. These modulators have a higher P-gp inhibiting capacity and deprived pharmacodynamic activity than the first-generation inhibitors. Further novel third-generation inhibitors are under development and intense study. Modulators such as OC144093, LY335979 and XR9576 were developed with the objectives of creating more potent, higher affinity inhibitors of P-gp. The third-generation modulators have about 10-fold higher efficacy than the second-generation inhibitors (Varma et *al.*, 2003).

Dexverapamil, which is the R-enantiomer of verapamil (a cardiovascular drug), retains the P-gp inhibiting activity of verapamil with less cardiotoxic effects (Plumb et *al.*, 1990; Hollt et *al.*, 1992). Despite the decreased toxicity of dexverapamil, previous studies have shown that the cardiovascular difficulties still occur at higher dosages used to attain a reversed P-gp function. Therefore its use is inadvisable for many patients (Bissett et *al.*, 1991).

Cyclosporin A was also considered a potential candidate for P-gp inhibition in clinical application. However, the immunosurpressive activity of cyclosoporin A restricted it's use in clinical oncology.

Further research led to the discovery of PSC 833, an analogue of cyclosporin A which lacks the immunosurpressive effects (Slater et *al.*, 1986; Twentyman , 1988). PSC 833 is a potent inhibitor of P-gp as well as a slowly transported substrate of P-gp (Fischer et *al.*, 1998). In a study, PSC833 was found to increase oral bioavailability of etoposide in

rats (Keller et *al.*, 1992). One difficulty associated with PSC 833 is its ability to also inhibit cytochrome P450 3A4(CYP3A4), the major drug-metabolizing enzyme in the body. Once administered to patients, it may slow down the metabolism of those drugs that are degraded by CYP3A4 and increase their toxic side effects. For this reason PSC833 cannot be used in high doses (Sikic, 1999; Schinkel and Jonker, 2003).

GF120918, an acridonecarboxamide derivative was also shown to be an effective P-gp modulator both *in vivo* and *in vitro* (Hya et *al.*, 1993). Unlike PSC833, this inhibitor does not appear to affect the clearance of drugs induced by drug-metabolizing enzymes and can be administered at higher dosage without apparent toxic side effects. GF120918 is also known to inhibit another ABC transporter, BCRP (Bruin et *al.*, 1999).

Other P-gp inhibitors, include LY335979, XR9576 and OC144093, which are highly efficacious and selective inhibitors with low toxicity (Dantzing, et *al.*, 1996; Mistry et *al.*, 2001; Newman et *al.*, 2000).

P-gp modulation can also be achieved through inhibition of ATP hydrolysis. Compounds such as quercetin, a naturally occurring flavonoid has shown to affect ATP hydrolysis by an unknown mechanism, and therefore inhibit P-gp function (Shapiro, 1997). Furthermore, surfactants such as Tween 80 and cremophor EL are also known to inhibit P-gp activity by alternating integrity of the membrane lipids. Studies by Hugger et *al.* demonstrated the ability of these pharmaceutical excipients to modify the fluidity of the cell membrane and increase of the accumulation of P-gp substrates in Caco-2 cell lines (Hugger et *al.*, 2002). These compounds could be used in low levels and therefore have less toxic side effects. Synthetic amphiphilic polymers such as poloxamers of the Pluronic® family also have the ability to modulate P-gp induced MDR, and this subject will be further discussed in chapter 1.7 of this thesis.

Table 1.4.3.1 List of P-gp inhibitors

First-generation Inhibitors	**References**
Verapamil (competitive inhibitor)	Tsuruo et *al.*, 1981
Cyclosporin A (competitive inhibitor)	Lum et *al.*, 1992
Second generation inhibitors	
PSC 833(Valspodar)	Keller et *al.*, 1992
R-verapamil	Tolcher et *al.*,1996
GF120918	Hyafil et *al.*, 1993
VX-710	Rowinsky et *al.*, 1998
MS-209	Kimura et *al.*, 2002
Third-generation inhibitors	
LY335979	Dantzig et *al.*,1996
OC144-093	Newman et *al.*, 2000
R101933	van Zuylen et *al.*, 2002
XR9576	Mistry et *al.*, 2001
Pharmaceutical excipients	
Tween 80	Zhang et *al.*, 2003
Cremophore	Webster et *al.*, 1993
Polyethylene glycol	Orlowski and Garrigos et *al.*, 1999

1.5 Multidrug resistance protein, MRP1

1.5.1 General properties and mechanism of MRP1

Multidrug resistance protein 1 (MRP1, also known as ABCC1) is a 190-kDa-membrane phosphoglycoprotein belonging to the ABC superfamily. The *MRP1* gene was first identified in doxorubicin resistant human lung carcinoma cell lines that did not overexpress P-gp (Mirski et *al.*, 1987; Cole et *al.*, 1992). The encoded protein consists of 1531 amino acids and was discovered by an antiserum raised against a synthetic peptide that corresponded to a reduced sequence homology within the P-gp NBF (Marquardt et *al.*, 1990). MRP1 shares about 15% amino acid identity with P-gp and confers resistance to a similar, however not identical spectrum of drugs (Hipfner et *al.*, 1999). Referred to as a glutathione-X conjugate pump (GS-X pump), MRP1 is involved in transporting anionic substrates (Borst and Elferink, 2002). Various orthologs of MRP1 from rabbit, bovine, rat, and mouse have been cloned and demonstrated a great degree of sequence conservation between species and with human MRP1 (van Kuijck *et al.*, 1996; Godinot *et al.*, 2003). MRP1 is a full-transporter characterized by a three-membrane-spanning domain (MSD) structure. In total, the protein is composed of seventeen transmembrane (TM) domains and two NBFs (Figure 1.5). The N-terminal region consists of MSD1, a ~200 amino acid domain in which five TM segments are present. The N-terminal region is connected to a P-gp-like core segment through a cytoplasmic loop. The core structure is composed of two hydrophobic MSDs (MSD2 and MSD3); each of them is composed of six TM domains and each one is followed by a single NBF (Cole et *al.*, 1992; Stride et *al.*, 1996; Kast and Gros, 1998). MRP1 topology studies so far indicate that glycosylation is not required for MRP1 transport function since its absence does not appear to demonstrate any major variation in substrate specificity (Hipfner et *al.*, 1997; Muller et *al.*, 2002). MRP1 is expressed in most tissues. In humans, it is highly expressed in the lung, kidney, testis, peripheral blood mononuclear cells and lateral membranes. (Cole et *al.*, 1992; Flens et *al.*, 1996).

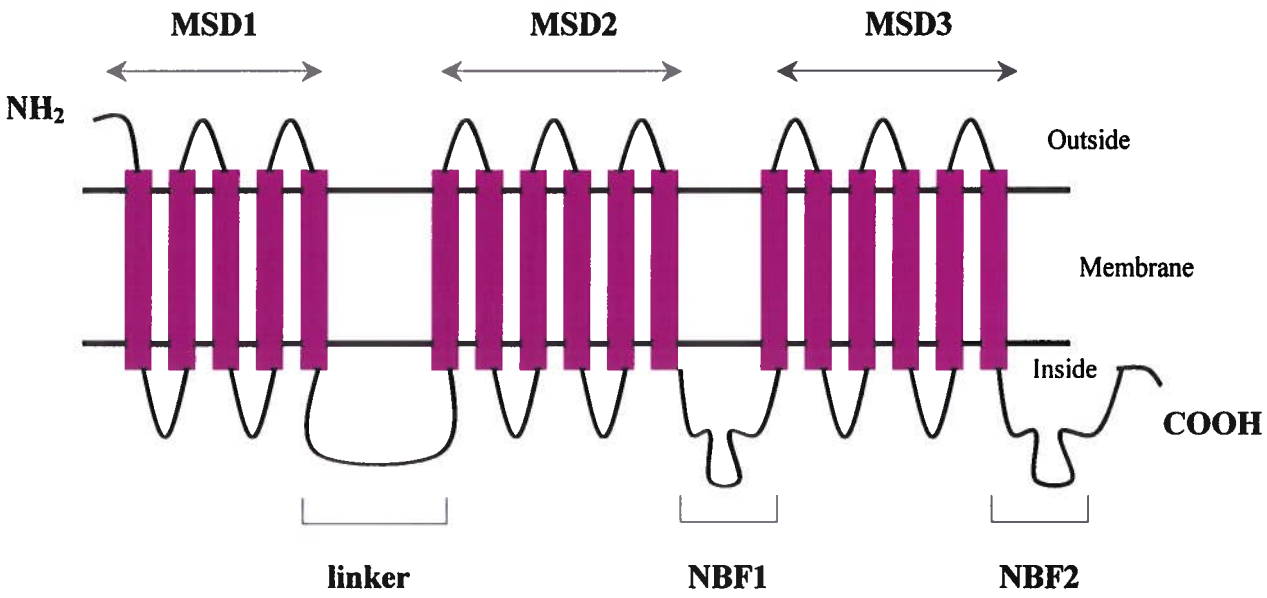


Figure 1.5 Membrane topology of Multidrug Resistance Protein 1 (MRP1)
MRP1 is a full-transporter characterized by a N-terminal membrane-spanning domain (MSD1) linked via a cytoplasmic loop to a P-gp-like core. The core contains two nucleotide-binding folds (NBFs) and two MSDs, each composed of six α-helical transmembrane (TM) segments. In total, MRP1 is comprised of 17 TM domains.
Adapted from (Hopper et *al.*, 2001).

The mechanism by which MRP1 mediates resistance to various substrates has yet to be elucidated. It has been suggested that MRP1 confers resistance by a mechanism similar but possibly also different than that used by P-gp. After various studies it has been proposed that once the drug enters the cell, MRP1 might mediate drug resistance based on various mechanisms: a) sequestration of the drug into intracellular vesicles; b) export of the drug from the cell via or without a co-transport mechanism with glutathione (GSH); or c) detoxification of drug metabolites through conjugation with GSH and followed by MRP1-mediated efflux (Van Luyn et *al.*1998; Loe et *al.*, 1998; Wang and Ballatori, 1998; Renes et *al.*, 2000).

In certain cell types, MRP1 was detected in the vesicle membranes possibly derived from Golgi apparatus. A study demonstrated that in drug resistant cells expressing this protein, the MRP1 substrate 2,4-dinitrophenyl chloride-glutathione was sequestered in these vesicles (Van Luyn et *al.*1998). MRP1 is an active transporter of GSH, oxidized GSH, glucuronides and sulfate conjugated organic anions (Leslie et *al.*, 2001; Deeley and Cole, 2002). In studies involving the transport of GSH-sulfate (GSH *S*) conjugates of the alkylating agents chloroambucil and melphalan, MRP1 was able to transport these compounds. However, MRP1 is not known to confer resistance to chloroambucil except when combined with elevated levels of GSTμ isozyme expression (Barnouin et *al.*, 1998; Morrow et *al.*, 1998; Horton et *al.*, 1999). Nevertheless, MRP1 confers resistance to various chemotherapeutic drugs, which are not substrates for GSHs such as doxorubicin and etoposide (O'Brien and Tew, 1996). However, GSH is assumed to play an essential role in MRP1-mediated drug resistance. Unlike P-gp, MRP1 is GSH–dependent for the transport of natural product drugs such as vincristine and daunorubucin (Loe et *al.*, 1996; Renes et *al.*, 1999). Intracellular metabolism of anticancer drugs such as doxorubicin results in formation of toxic metabolites like alpha and beta-unsaturated aldehyde 4-hydroxynonenal (Ollinger and Brunmark, 1994). These toxic metabolites can be detoxified by conjugating with GSH and can be extruded from the cell by MRP1 (Renes et *al.*, 2000).

The exact mechanism for drug-induced MRP1-expression is still unclear. However evidence suggests that the oxidative stress induced by tumour necrosis factor-alpha, a hormone -like protein can generates this expression (Stein et *al.*, 1997).

1.5.2 Physiological roles of MRP1

MRP1 is linked to the protection of cells against xenobiotics. One of the main physiological functions of MRP1 is the efflux of anionic conjugates, especially GSH *S*-conjugates (Muller et *al.*, 1994). In cells, GSH plays a critical role in detoxification of reactive oxygen intermediates, heavy metal oxyanions and electrophiles via reduction or conjugation (Wang and Ballatori, 1998). GSH *S*-conjugates are substrates with high affinity for MRP1, and the affinity increases depending on the length of the alkyl group (Loe et *al.*, 1996). Hence, MRP1 plays a major role in the transport of these substrates. MRP1 is also assumed to be involved in activating cellular defence against oxidative stress that can be caused in presence of xenobiotics. A study demonstrated that MRP1 function is necessary for activation of T-helper 1 cells, which are involved in regulation and activation of the immune response (Prechtl *et al.*, 2000). Other studies have also shown the importance of MRP1 in protective cellular function; mice lacking MRP1 showed increased sensitivity to etoposide accompanied with greater toxicity for cells (Lorico et *al.*, 1997; Wijnholds et *al.*, 1998).

1.5.3 Substrates and inhibitors of MRP1

MRP1 is capable of transporting a broad array of compounds with vast structural diversity. MRP1 is a principal active transporter of amphipathic organic anions like GSH, glutathione disulfide, and many endogenously formed GSH conjugates. For example, the inflammatory mediator leukotriene C_4 (LTC_4) as well as various glucuronide and sulphate conjugates (Leslie et *al.*, 2001). The resistance profile of MRP1 is similar but not identical to P-gp. MRP1 confers resistance to numerous cytotoxic drugs and their metabolites such as: anthracylines (doxorubucin, daunorubicin, epirubicin); epipodophyllotoxins (etoposide); vinca alkaloids (vincristine, vinblastine) and camptothecins (CPT-11, SN-38) (Hipfner et *al.*, 1999; Borst et *al*, 2000). The transport of certain non-anionic anti-cancer drugs by MRP1 requires reduced GHS supply (Zaman et *al.*, 1995). The exact mechanistic role of GSH in MRP1- mediated transport is still not fully explained. While facilitating the export of several MRP1 substrates, GSH could be also involved in enhancing the potency of some MRP1 inhibitors (Loe et *al.*, 1996; Renes et *al.*, 1999). Further, MRP1 confers

resistance to heavy metal oxyanions like arsenite, antimony- GSH complexes and several fluorescent probes including fluorescein, fluo-3, calcein and pH indicator BCECF (2', 7' - bis(2-carboxyethyl)-5(6)-carboxyfluorescein) (Zaman et *al.*, 1995; Hollo et *al.*, 1996; Feller et *al.*, 1995). Various MRP1 substrates are listed in Table 1.5.3 (by Renes et *al.*, 2000).

Table 1.5.3 List of MRP1 substrates

Anticancer drugs	**References**
Daunorubicin	Cole *et al.*, 1994
Doxorubucin	Grant *et al.*, 1994
Epirubicin	Cole *et al.*, 1994
Etoposide	Grant *et al.*, 1994
Vincristine	Grant *et al.*, 1994
Vinblastine	Cole *et al.*, 1994
7-ethyl-10-hydroxy-camptothecin (SN-38)	Chen *et al.*, 1999
CPT-11	Chen *et al.*, 1999
Glutathione (GSH) conjugates	
Leukotriene C_4 (LC_4)	Leier *et al.*, 1994
Leukotriene D_4 (LD_4)	Leier *et al.*, 1994
Leukotriene E_4 (LE_4)	Leier *et al.*, 1994
N-Acetyl-leukotriene E_4	Jedlitschky *et al.*, 1996
S-glutathionyl 2.4-dinitrobenzene	Muller *et al.*, 1994
S-glutathionyl prostaglandin A_1	Evers *et al.*, 1997
S-glutathionyl prostaglandin A_2	Evers *et al.*, 1997
S-glutathionyl aflatoxin B_1	Loe *et al.*, 1997
S-glutathionyl ethacrynic acid	Zaman *et al.*, 1996
S-glutathionyl N-ethylmaleimide	Bakos *et al.*, 1998
S-glutathionyl 4-hydroxynonenal	Renes *et al.*, 2000
Monochloro-monoglutathionyl melphalan	Barnouin *et al.*, 1998
Monohydroxy-monoglutathionyl melphalan	Barnouin *et al.*, 1998
Monochloro-monoglutathionyl chlorambucil	Barnouin *et al.*, 1998
Monohydroxy-monoglutathionyl chlorambucil	Barnouin *et al.*, 1998
Bisglutathionyl chlorambucil	Barnouin *et al.*, 1998
Glucuronide conjugates	
Bisglucuronosyl bilirubin	Jedlitschky et *al.*, 1997
Monoglucoronosyl bilirium	Jedlitschky et *al.*, 1997
6α-Glucuronosyl hyodeoxycholate	Jedlitschky et *al.*, 1996
17β-Glucuronosyl estradiol	Jedlitschky et *al.*, 1996
Glucuronosyl etoposide	Jedlitschky et *al.*, 1996
Sulphate conjugates	
3α-Sulfatolithocholyl taurine	Jedlitschky et *al.*, 1996
Esterone 3-sulfate	Qian et *al.*, 2001
Dehydroepiandrosterone 3-sulfate(DHEAS)	Zelcer et *al.*, 2003
Fluorescent probes	
Fluorescein	Sun et *al.*, 2001
Fluo-3	Keppler *et al.*, 1999
Calcein	Feller *et al.*, 1995
2',7' -bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)	Draper *et al.*, 1997
Other	
Glutathione (GHS)	Zaman *et al.*, 1995
Glutathione disulfide	Leier *et al.*, 1996
Sodium arsenite	Cole *et al.*, 1994
Sodium arsenate	Cole *et al.*, 1994
Potassium antimonite	Cole *et al.*, 1994
Potassium antimony tartrate	Cole *et al.*, 1994

Inhibitors of MRP1 can be divided into three different classes (Table 1.5.3.1). The first group comprises non-specific MRP1 inhibitors that modulate the activity of other transporters such as: organic anion transporters that are not members of the ABC superfamily, as well as ATP-dependent transporters like P-gp. These inhibitors are usually non-specific, however, some specificity may actually be achieved, depending on their inhibitory potency and the type of transporter they are modulating. Their mechanism of inhibition is still not known. Inhibitors of organic anion transporters, which modulate MRP1 activity, include sulfinpyrazone, indomethacin and probenecid (Roller et *al.*, 1999; Gollapudi et *al.*, 1997). Compounds having dual acting capacity that modulate the transport activity of other ABC transporters such as P-gp as well as MRP1 include: dihydropyridine (PAK-104P), VX-710 (Bricodar), cyclosporin A, verapamil, agosterol A, flavonoids like genistein, biochanin A and quercetin as well as some steroid derivatives like budesonide and RU484 (Sumizawa et *al.*, 1997; Germann et *al.*, 1997; Barrand et *al.*, 1993; Aoki et *al.*, 2001; Nguyen et *al.*, 2003; Hooijberg et *al.*, 1997; Payen et *al.*, 1999; Bandi and Kompella, 2002).

The second class of modulators that are more specific to MRP1-mediated transport includes the LTD_4 antagonist MK571, the peptide leukotriene receptor antagonist ONO-1078 (Gekeler *et al.*, 1995; Nakano *et al.*, 1998). In addition, several peptidomimetic GSH-conjugates analogues have also shown to be potential MRP1 inhibitors (Burg *et al.*, 2002).

Third class modulators, which are more potent, and highly specific modulators of MRP1 transport activity have also been developed. These inhibitors are LY475776 and LY402913, which are tricyclic isoxazoles that inhibit MRP1-mediated LTC_4 transport in a GSH-dependent manner (Qian et *al.*, 2002; Norman et *al.*, 2002).

Table 1.5.3.1 List of MRP1 inhibitors

Non-specific transport inhibitors	**References**
Sulfinpyrazone	Bakos et *al.*, 2000
Indomethacin	Roller et *al.*, 1999
Probenecid	Gollapudi et *al.*, 1997
PAK-104P	Sumizawa et *al.*, 1997
VX-710	Germann et *al.*, 1997
Cyclosporin A	Barrand et *al.*, 1993
Verapamil	Barrand et *al.*, 1993
Agosterol A	Aoki et *al.*, 2001
Quercetin	Nguyen et *al.*, 2003
Genistein	Hooijberg et *al.*, 1997
Budesonide	Bandi and Kompella, 2002
RU486	Payen et *al.*, 1999
MRP-specific inhibitors	
MK571	Gekeler et *al.*, 1995
Glibenclamide	Payen et *al.*, 2001
ONO-1078	Nakano et *al.*, 1998
Peptidomimetic GSH-conjugates	Burg et *al.*, 2002
GHS-dependent inhibitors	
LY475776	Qian et *al.*, 2002
LY402913	Norman et *al.*, 2002

1.6 Breast cancer resistance protein, BCRP

1.6.1 General properties and mechanism of BCRP

Human breast cancer resistance protein (BCRP, also known as ABCG2, MXR and ABC-P) was cloned from a highly doxorubucin-verapamil resistant MCF-7 breast cancer cell line (MCF-7/AdrVp) that did not express P-gp or MRP1 (Doyle et *al.*, 1998; Lee et *al.*, 1997). Concurrently, human BCRP was also cloned independently by two other laboratories, and named MXR (discovered in mitoxantrone-resistant human colon carcinoma cell lines S1-M1-80) and ABC-P (human placenta ABC protein having identical sequence to BCRP cDNA) (Miyake et *al.*, 1999; Allikmets et *al.*, 1998). Since BCRP, MXR and ABC-P is basically the same protein with minor differences in amino acid sequence, we will use the original name BCRP throughout this thesis. Shortly after, mouse *Bcrp1* was also cloned showing 86% similarities to human *BCRP* (Allen and Schinkel 2002; Allen et *al.*, 1999). BCRP cDNA transfection studies revealed that this protein can confer resistance to doxorubicin, mitoxantrone, daunorubicin and efflux of its substrates occurs in an energy-dependent manner (via ATP-hydrolysis) (Ozvegy et *al.*, 2001; Doyle et *al.*, 1998).

BCRP is a 655 amino acid ABC protein and is considerably smaller then P-gp and MRP1. The protein is made of one nucleotide-binding fold (NBF) at the N-terminal followed by one membrane-spanning domain (MSD) at C-terminal end that harbours six putative transmembrane domains (TM) for substrate binding (Doyle and Ross, 2003). Unlike P-gp and MRP1, a unique feature of BCRP is that NBF precedes MSD in the domain rearrangement (Figure 1.6). BCRP is a half-transporter and possibly functions as a homodimer by dimerizing with another half transporter to form a functional transporter. BCRP is glycosylated, yet it is unclear if this modification contributes to its transport activity (Litman et *al.*, 2002). BCRP is mainly localized to the plasma membranes of mammalian cells (Rocchi et *al.*, 2000). In normal human tissues, BCRP is highly expressed in the placenta, prostate, epithelium in the small intestine, liver canalicular membrane, colon and ovary (Doyle et *al.*, 1998). BCRP is also expressed in numerous types of human cancers including hematological carcinoma, hepatocellular cancer, gastric carcinoma, colon cancer, small cell lung cancer and melanoma (Diestra et *al.*, 2002). Even though

BCRP was originally isolated from a human breast cancer cell line, there is no evidence at present to indicate that BCRP is preferentially expressed in breast cancer cells or whether it plays a significant a role in drug resistance in breast carcinoma (Kanzaki et *al.*, 2001; Burger et *al.*, 2003). The exact mechanism by which BCRP is able to efflux various substrates have not yet been fully explained. A study by Özvegy *et al.*, reported high levels of vanadate-sensitive ATPase activity in isolated membrane preparations of Sf9 insect cells overexpressing BCRP when stimulated by doxorubucin, daunorubicin, mitoxantrone, prazosin and rhodamine 123. However, BCRP-ATPase activity in Sf9 membranes was inhibited in the presence of Na-orthovanadate, N-ethylmeleimide and cyclosporin A (Ozvegy *et al.*2001). Another study also demonstrated photolabelling of 8-azido [α^{32}-P] ATP with Sf9 cells overexpressing BCRP in the presence of vanadate, Mg^{2+} or Co^{2+}, allowing the investgation of its membrane ATPase activity. The study conclude that BCRP is able to hydrolyse ATP and functions as an ATP-dependent efflux pump (Ozvegy et *al.*,2002). On the other hand, Sf9 cells expressing wild-type BCRP (containing arginine at position 482) and its mutant variants (R482G, R482T) as well as catalytic center mutant (K86M) have indicated that K86M had transport effect or ATP hydrolytic activity. However, the wild-type BCRP and its variants R482G and R482T showed different drug transport activities (Ozvegy *et al.*, 2002). Present studies on BCRP structure, function and transport mechanisms are still at an early stage. Further studies are needed to better understand this transporter and to assess its importance in drug resistance.

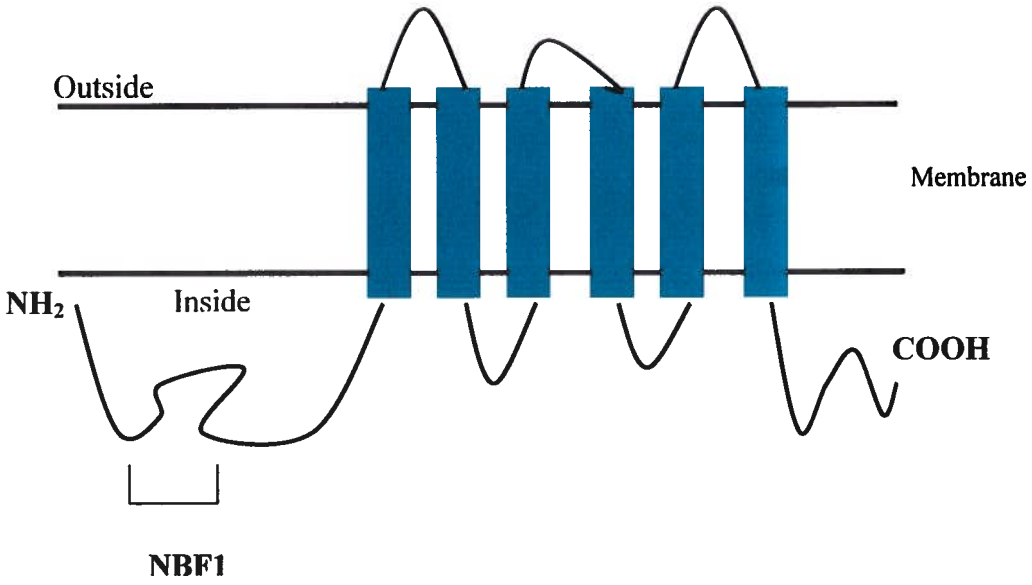


Figure 1.6 Membrane topology of Breast Cancer Resistance Protein (BCRP)
BCRP is a half transporter and possibly functions as a homodimer bridged by disulfide bonds. BCRP contains one nucleotide-binding fold (NBF1) followed by one membrane-spanning domain (MSD) with six α-helical transmembrane (TM) segments. This is the only known ABC human protein with a domain rearrangement in which the NBF precedes MSD, which is a unique feature of this protein.
Adapted from (Doyle and Ross, 2003).

1.6.2 Physiological roles of BCRP

To date, physiological functions of BCRP are not fully elucidated. Several groups are currently exploring the roles of this transporter in various tissues. BCRP is known to play a protective role in the maternal-fetal barrier of the placenta against the entry of topotecan (Jonker et *al.*, 2000). In addition, an investigation involving BCRP-deficient mice showed an increase in sensitivity to the toxic effect of mitoxantrone (Zhou et *al.*, 2002). These observations suggest that BCRP is involved in a cellular defence mechanism role against toxic compounds similar to that of P-gp. Another possible physiological function of BCRP involves the high expression of murine Bcrp1 and human BCRP in "side population" cells that are enriched by stem cells in which low accumulation of the Hoechst 33342 dye were observed (Zhou et *al.*, 2001; Scharenberg et *al.*, 2002). Human BCRP is known to efflux Hoechst 33342; therefore it is very possible that the high-level murine and human Bcrp1/BCRP expression in side population cells is responsible for excluding the uptake of this dye.

1.6.3 Substrates and inhibitors of BCRP

Like P-gp and MRP1, BCRP is able to transport a wide range of substrates from chemotherapeutic drugs to organic anion conjugates. BCRP displays a different substrate specificity that also overlaps the P-gp and MRP1 substrate profiles (refer to Table 1.6.3). BCRP can transport substrates including: anthracyclines (doxorubicin, daunorubicin); anthracenes (mitoxantrone, bisantrene); camptothecin derivatives (topotecan, irinotecan and its active metabolite SN-38); cytotoxic agents (prazosin, indolocarbazole); diagnostic probes (Rhodamine 123, Hoechst 33342) and conjugated organic anions (estrone -3-sulfate, 17ß-estradiol 17-[ß-D-glucuronide]) (Litman et *al.*, 2000; Naktomi et *al.*, 2001; Maliepaard et *al.*, 2001; Robey et *al.*, 2001; Scharenberg et *al.*, 2002; Chen et *al.*, 2003). The structural requirement for recognition and transport of camptothecin analogues by BCRP is not fully understood. Two known substrates of BCRP, topotecan and SN-38 possess a hydroxyl group at the 10^{th} position of the camptothecin A-ring. A study by Rajendra et *al.*, has reported that BCRP confer resistance to 9-aminocamptothecin but not 9-nitrocamptothecin, therefore suggesting that polar groups at the 9^{th} or 10^{th} position of the

camptothecin A-ring is involved in substrate recognition by BCRP (Rajendra et *al.*, 2003). Another study by Yoshikawa et *al.* also showed that BCRP had more affinity to camptothecin analogues with high polarity than analogues with low polarity (Yoshikawa et *al.*, 2004). A more detailed list of BCRP substrates are summarized in Table 1.6.3 (by Mao and Unadkat, 2005).

Table 1.6.3 List of BCRP substrates

Anthracyclines	**References**
Doxorubicin	Litman et *al.*, 2000
Daunorubicin	Litman et *al.*, 2000
Epirubicin	Litman et *al.*, 2000
Anthracenes	
Mitoxantrone	Litman et *al.*, 2000
Bisantrene	Litman et *al.*, 2000
Camptothecin analogues	
SN-38 (7-ethyl-10-hydroxycamptothecin)	Nakatomi et *al.*, 2001
Topotecan	Litman et *al.*, 2000
Irinotecan	Maliepaard et *al.*, 2001
9-aminocamptothecin	Rajendra et *al.*, 2003
Diflomotecan	Sparreboom et *al.*, 2004
Methotrexate	Volk et *al.*, 2002
Nucleoside analogues	
Zidovudine (AZT)	Wang et *al.*, 2003
Lamivudine	Wang et *al.*, 2003
Cytotoxic agents	
Prazosin	Litman et *al.*, 2000
Indolocarbazole	Nakagawa et *al.*, 2002
Indolocarbazole topoisomerase I inhibitors (NB-506, J-107088)	Komatani et *al.*, 2001
Pantoprazole	Breedveld et *al.*, 2004
Imatinib mesylate (STI571)	Burger et *al.*, 2004
Flavopiridol	Robey et *al.*, 2001
Diagnostic Dyes	
Rhodamine 123	Robey et *al.*, 2001
Lysotracker Green	Robey et *al.*, 2001
Aza-anthrapyrazole (BBR 3390)	Rabindran et *al.*, 2000
Hoechst 33342	Scharenberg et *al.*, 2002
BODIPY-Prazosin	Litman et *al.*, 2000
Others	
17ß-estradiol 17-(ß-D-glucuronide)	Chen et al., 2003
E3040G	Suzuki et *al.*, 2003
E3040S	Suzuki et *al.*, 2003
4-MUS	Suzuki et *al.*, 2003
4-MUG	Suzuki et *al.*, 2003
Genestein	Imai et *al.*, 2004
Phosphatidylserine	Woehlecke et *al.*, 2003
GV196771	Polli et *al.*, 2004

Inhibitors of BCRP include various compounds summarized in Table 1.6.3.1 (by Mao and Unadkat, 2005). However, whether any of these compounds are clinically useful to inhibit BCRP-mediated drug resistance is still under study. GF120918, a second-generation P-gp inhibitor is one of the most potent inhibitors of BCRP. The natural product Fumitremorgin C, a tremorgenic mycotoxin produced by the fungus *Aspergillus fumigatus* is another BCRP inhibitor showing significant increase in the toxicity of mitoxantrone and topotecan in BCRP-overexpressing cells. Compared to GF120918, Fumitremorgin C is a relatively specific inhibitor of BCRP. It shows no inhibitory effect on P-gp and MRP-mediated drug resistance; it exhibits neurotoxicity side effects (Rabindran et *al.*, 1998; Rabindran et *al.*, 2000). Furthermore, Fumitremorgin C analogues Ko132 and Ko 134 have greater activity than Fumitremorgin C with lower toxicity (van Loevezijn et *al.*, 2001). HER (human epidermal growth factor receptor) tyrosine kinase inhibitors (TKI) specifically inhibiting cellular signalling pathways are well known inhibitors of BCRP. These compounds include CI1033 (PD 183805), iressa (structure similar to CI1033) and EKI-785. CI1033 increases the accumulation of topotecan and SN-38 in cells transfected with BCRP, but not on cells transfected with empty vector; therefore suggesting that CI1033 is also a substrate of BCRP (Erlichman et *al.*, 2001). In contrast to CI1033, iressa is not a substrate of BCRP (Ozvegy-Laczka et *al.*, 2004). Other inhibitors of BCRP include the pipecolinate derivative VX-710; HIV protease inhibitors (ritonavir, nelfinavir and saquinavir); a coumermycin antibiotic novobiocin; rauwolfia alkaloid reserpine (also an inhibitor of P-gp); estrogen antagonists (tamoxifen and its derivatives TAG-11, TAG-139) and food dietary flavonoids (chrysin and biochanin A) (Mindermann et *al.*, 2004; Gupta et *al.*, 2004; Shiozawa et *al.*, 2004; Zhang et *al.*, 2004; Sugimoto et *al.*, 2003).

Table 1.6.3.1 List of BCRP inhibitors

Second generation inhibitors	**References**
GF120918	Allen et *al.*, 1999
VX-710 (Bricodar)	Minderman et *al.*, 2004
Mycotoxin inhibitors	
Fumitremorgin C	Rabindran et *al.*, 2000
Ko132	van Loevezijn et *al.*, 2001
Ko134	van Loevezijn et *al.*, 2001
HER tyrosine kinase inhibitors	
Iressa (ZD1839 or Gefitinib)	Ozvegy-Laczka et *al.*, 2004
CI1033	Erlichman et *al.*, 2001
EKI-1-785	Ozvegy-Laczka et *al.*, 2004
HIV Protease Inhibitors	
Ritonavir	Gupta et *al.*, 2004
Nelfinavir	Gupta et *al.*, 2004
Saquinavir	Gupta et *al.*, 2004
Others	
Novobiocin	Shiozawa et *al.*, 2004
Estrone	Sugimoto et *al.*, 2003
Tamoxifen	Sugimoto et *al.*, 2003
TAG-11	Sugimoto et *al.*, 2003
TAG-139	Sugimoto et *al.*, 2003
Reserpine	Ebert et *al.*, 2005
Chrysin	*Zhang* et *al.*, 2004
Biochanin A	*Zhang* et *al.*, 2004

1.7 Poloxamers of the Pluronic® family

Drug delivery and drug targeting systems based on synthetic polymers have many important applications in the development of novel drug therapies. One particularly important group of polymers in medical applications are poloxamers, block copolymers serving as drug carriers and as biological response modifying agents, that have shown promising results in enhancing drug performance for drug and gene delivery (Kabanov et *al.*, 1992; Alakhov and Kobanov 1998; Lemieux et *al.*, 2000). Poloxamers are commercially available from BASF corporation under the registered trade name Pluronics®. They are used for example as non-toxic emulsifiers for perfluoroganic oxygen carriers in artificial blood formulations (Lowe and Armstrong, 1990). In addition, Pluronic® block copolymers also sensitize MDR cells by inhibiting certain drug efflux transporters (Alakhov et *al.*, 1996; Venne et *al.*, 1996).

1.7.1 Structure and physicochemical characteristics of Pluronics®

Pluronics® are synthetic amphiphilic block copolymers of low toxicity. They are composed of a block of poly(propylene oxide; PO) at the centre and of two blocks of poly(ethylene oxide; EO) arranged in a basic A-B-A structure. Molecular weights of Pluronics® range from 1kD to 13kD, while the molecules differ in the number of PEO (n) and PPO (m) units, and as a result differ in hydrophilic-lipophilic balance (HLB). In aqueous solution, Pluronic® unimers at or above a certain concentration called the critical micelle concentration (CMC), start to aggregate and self-assemble, and form micelles. The CMC strongly depends on the length of PEO and PPO segments. Generally, an increase in the lengths of hydrophobic segments results in CMC decrease (Alexandris et *al.*, 1994). CMC is also temperature-dependent. The CMC is decreases with an increase in temperature, and consequently at lower temperatures Pluronics® may exist in unimer form, and although temperatures may exceed the critical micellization temperature (cmt) they start to self-assemble into micelles. This is mainly caused by the differential effects of temperature on dehydration of the PPO or PEO blocks (Zhou and Chu, 1987; Alexandris et *al.*, 1994). Micelles are spherical colloidal aggregates of surfactant molecules of 100nm in

size. They are composed of hydrophobic core made of PPO and surrounded by hydrophilic PEO shell that helps to stabilize the micelle.

Pluronic® micelles can dissolve hydrophobic substances. The distribution of a substance to be solubilized in micelles depends very much on its polarity. Non-polar molecules solubilize in the micellar hydrophobic core, while molecules with intermediate polarity are distributed somewhere between the hydrophobic *head* and hydrophilic *tails* of the micelle, and polar molecules solubilize in the hydrophilic surroundings. The solubilization capacity of micelles towards a compound is described by P, the partitioning coefficient, which is the ratio of concentrations of the substance in the micelle vs. the concentration in the surrounding aqueous phase in equilibrium (Figure 1.7) (Attwood and Florence, 1983).

The CMC of polymers can be measured by surface tension measurements (by ring-tearing technique using torsion weights with a thermostatic cell unit) and by fluorescent probes for example, (pyrene or 1,6-diphenyl-1,3,5-hexatriene). Fluorescence of these probes is dependent on the environment and increases upon incorporation into micelles (Kabanov et *al.*, 1995).

Pluronics® have very important pharmaceutical applications. Many drugs have low water solubility, which can be increased by the use of Pluronics® by favoring the formation of colloidal solutions, with the drug incorporated in micelles. This often enhances the bioavailability of the drugs administered orally.

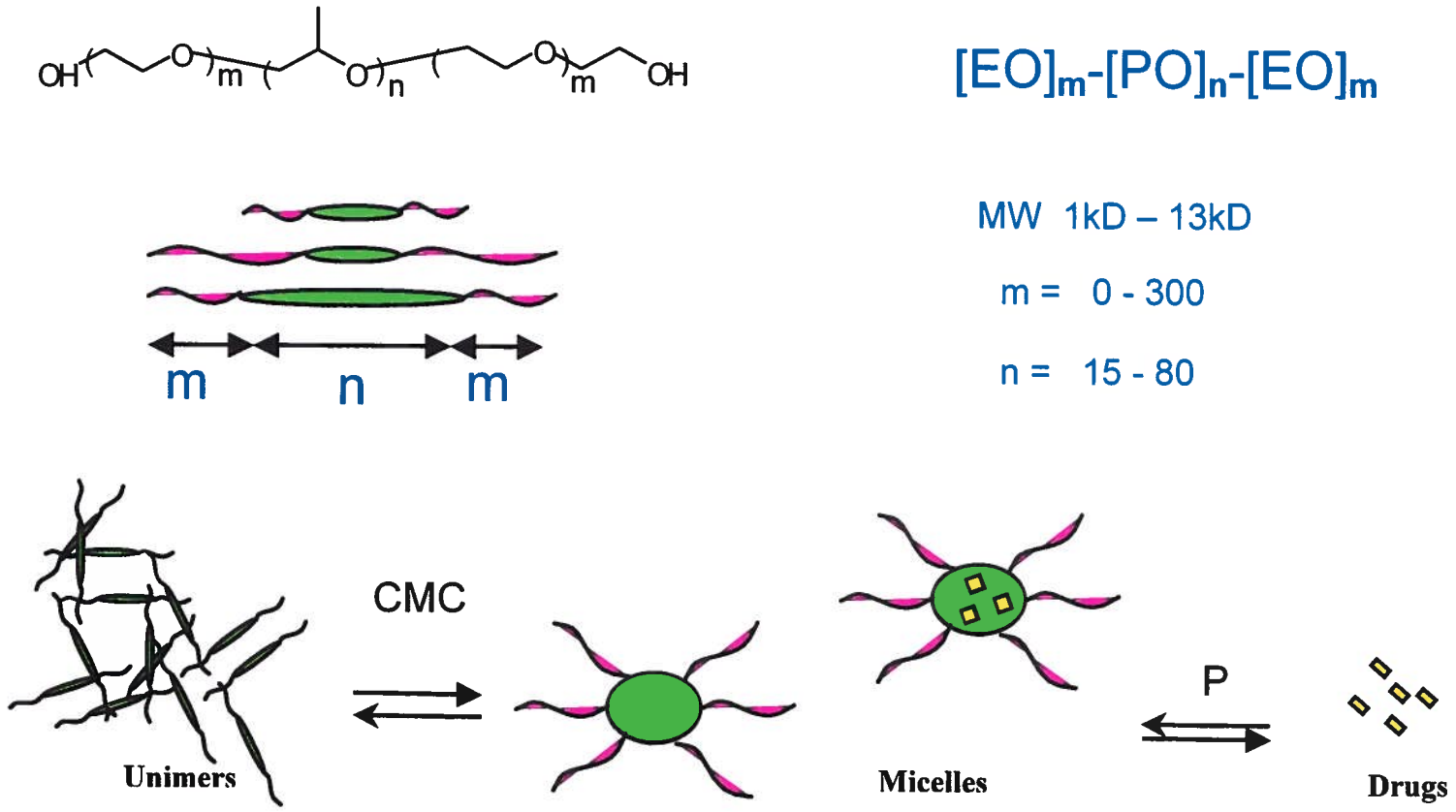


Figure 1.7 Physicochemical characteristics of Pluronics® and reversible polymeric unimer-micelle formation
Polymers of the Pluronic® family consist of a hydrophobic unit polypropylene oxide (PPO) and of two hydrophilic units polyethylene oxide (PEO). They vary in the number of PPO (n) and PEO (m) units and therefore differ in molecular weight ranging from 1kD to 13 kD. In aqueous solution, Pluronics® at or above critical micelle concentration (CMC), unimers (individual polymer molecules) start to aggregate and form micelles. The transition from unimer to micelle is concentration and temperature-dependent. Micelles solubilize hydrophobic molecules in aqueous environments and the partitioning coefficient (P) is an important parameter that characterizes drug distribution between micelles and the aqueous phase under equilibrium conditions. Source: Collette J.H. Poloxamers in: Handbook of Pharmaceutical Excipients 2nd edition, 1994.

1.7.2 Pluronics® as a sensitizing agent of MDR cells

Previous *in vitro and in vivo* studies have demonstrated the ability of some Pluronics® to sensitize MDR cancer cells overexpressing P-gp. These polymers increased the cytotoxic activity of antracycline antibiotics and other anti-tumour drugs by two to three orders of magnitude (Alakhov et *al.*, 1996; Venne et *al.*, 1996, Batrakova et *al.*, 1996). Particularly, Pluronic® P85 was shown to inhibit the P-gp transporter and improved the permeability of drugs across the blood brain barrier (Batrakova et *al.*, 2003). Studies have revealed the sensitizing effect of Pluronics® on MDR cells overexpressing P-gp, while the concentrations of the polymer were below or around its CMC. That demonstrates that Pluronic® unimers were responsible for the inhibition of the P-gp mediated drug efflux system, not the micelles (Batrakova et *al.*, 1998). The exact mechanism by which Pluronics® are able to sensitize MDR cells and inhibit ABC drug efflux transporters is still unclear. One possible mechanism has been suggested based on the studies by Batrakova et *al.* on bovine brain microvessel endothelial cells (BBMEC), an *in vitro* model of blood-brain barrier (Batrakova et *al.*, 2003). In these studies a wide range of Pluronics® differing in hydrophilic-lipophilic balance (HLB) have been tested. The lipophilic Pluronics® with intermediate length (30 to 60 units) and having HLB less than 20 were the most effective in inhibiting P-gp efflux in BBMECs (Figure 1.7.1). Pluronics® incubated with BBMECs overexpressing P-gp, caused fluidization of cellular membranes, leading to a decrease in Pgp ATPase activity. These effects were due to changes in the lipid microenvironment and inhibition in ATP synthesis resulting in ATP energy depletion that is essential for proper functioning of P-gp (Batrakova et *al.*, 2001a; Batrakova et *al.*, 2003). Inhibition in ATP synthesis is related to co-localization of Pluronics® in the mitochondria, and a decrease of the activity of electron transport chains (Rapoport et *al.*, 2000).

Thus far, few studies have demonstrated that Pluronic® P85 inhibits MRP1 or MRP2 drug efflux systems. Despite the differences in substrates and inhibitors of MRP and P-gp efflux systems, there were similarities detected in the effect of Pluronics® on sensitizing cells overexpressing these transporters. These similarities were: membrane fluidization, inhibition of MRP ATPase activity and enhancement of cytotoxicity of several drugs by Pluronic® P85. In addition, Pluronic® P85 exposed to cells overexpressing

MRP also reduced the GSH intracellular levels and GST activities (Miller et *al.*, 1999; Batrakova et *al.*, 2003). Studies evaluating the effect of Pluronics® on MRP-mediated drug transport are still at an early stage. A list of Pluronics® used in this study are presented in Table 1.7.

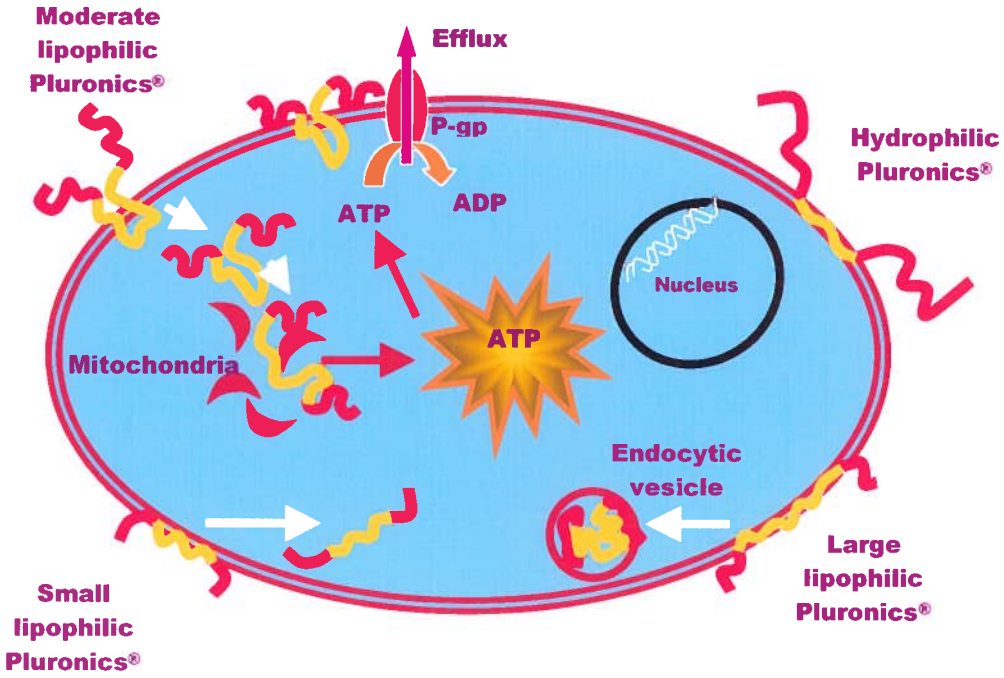


Figure 1.7.1 Interaction of Pluronic® copolymers with bovine brain microvessel endothelial cells (BBMEC)

A range of Pluronics® from hydrophilic to more lipophilic were tested on BBMEC. Lipophilic Pluronics® with intermediate PPO blocks such as Pluronics® L64, P85, L81 and P105 rapidly incorporate into the cell membrane resulting in an increase of membrane fluidization. Polymers reaching the mitochondria cause a decrease of Pgp ATPase activity due to changes in the lipid microenvironment and inhibition of ATP synthesis due to decreases in the activity of the electron transport chain.

Adapted from (Batrakova et *al.*, 2003).

Table 1.7 Structural characteristics of various Pluronic® polymers

BASF Pluronic® polymers	MW[a] (mol.wt.)	m[b]	n[c]	% EO/100[d]	HLB[e]
L10	3200	0	55	0	14
L31	1100	1	17	0.1	5
L35	1900	11	16	0.5	19
F38	4700	38	17	0.7	25
L43	1850	6	22	0.3	12
L44	2200	10	23	0.4	16
L61	2000	2	31	0.1	3
L64	2900	13	30	0.4	15
F68	8400	76	29	0.8	29
L81	2750	3	43	0.1	2
P84	4200	19	43	0.4	14
P85	4600	26	40	0.5	16
F87	7700	61	40	0.7	24
F88	11400	104	39	0.8	28
L92	3650	8	50	0.2	6
F108	14600	133	50	0.8	27
L101	3800	4	59	0.1	1
P104	5900	27	61	0.4	13
L121	4400	5	68	0.1	1
P123	5750	20	69	0.3	8
F127	12600	100	65	0.7	22

[a] Molecular weight of polymer provided by the manufacturer.
[b] Number of blocks of ethylene oxide (EO) units calculated using the molecular weights.
[c] Number of blocks of propylene oxide (PO) units calculated using the molecular weights.
[d] Percentage of ethylene oxide divided by 100 calculated using the molecular weight and number of EO.
[d] Hydrophilic/lipophilic balance of polymer. Determined by the manufacturer.

1.8 Experimental model

1.8.1 Cell lines

The MDR phenotype can be induced in many human and rodent cell lines by a gradual increase in concentration of certain cytotoxic agents in the culture medium. Such cells may overexpress ATP-dependent transmembrane proteins that are implicated in active efflux systems. The cells may originate from various tissues, and their MDR phenotypes may vary significantly. Several such selected cell lines are suitable for *in vitro* studies to determine the inhibitory effect of Pluronics® on active efflux systems. In the work presented herein we will be using as the experimental model the cell lines MCF-7 ADR, MCF-7 TPT and HL 60 ADR and their respective sensitive parental cell lines.

The MCF-7 ADR cells are derived from malignant adenocarcinomas resected from a patient during a surgery (Soule et *al.*, 1973). The cells were selected through exposure to Adriamycin® (commercial name for doxorubicin) that conferred the MDR phenotype and induced expression of high levels of P-gp (Batist et *al.*, 1986; Fairchild et *al.*, 1987; Moscow et *al.*, 1989). The expression of the *P-gp* gene in cells was analysed using quantitative reverse transcription-polymerase chain reaction (RT-PCR) demonstrating a 64-fold increase in *P-gp* gene expression on MCF-7 ADR cells relative to MCF-7 Adriamycin® sensitive parental cells normalized to 1-fold (communication by A. Semov, Supratek Pharma).

The MCF-7 TPT (topotecan resistant) breast cancer cell line was selected by the exposure of MCF-7 cells to an increasing concentration of topotecan, conferring MDR phenotype and overexpression of BCRP. The measurement of *BCRP* and *P-gp* expression was carried out on MCF-7 TPT and showed 16-fold increase of *BCRP* expression compared to 3.6-fold increase of *P-gp* expression relative to MCF-7 topotecan sensitive parental cells normalized to 1-fold for both genes (communication by A. Semov, Supratek Pharma).

The HL 60 ADR cells were derived from human promyelocyte leukemia. The cells were isolated by subculturing in progressively higher concentrations of Adriamycin® that caused overexpression of the *MRP1* gene (Bhalla et *al.*, 1985; Krishnamachary, 1993). The confirmation of *MRP1* overexpression on HL 60 ADR cells and the normal levels of

expression by HL 60 Adriamycin® sensitive parental cells was received from Dr. Alexander V. Kabanov.

1.8.2 Diagnostic probes and cytotoxic agents

The inhibitory effect of Pluronics® on P-gp-, MRP1- and BCRP-mediated drug efflux was studied in a series of experiments in which accumulation of drugs and probes in cells was evaluated in the presence of Pluronics® and other inhibitors.

The cytotoxic drugs/diagnostic probes used in this study with their chemical structures and their basic features are listed in Table 1.8 and are described as follows:

Rhodamine 123: Rhodamine 123 (R123) is a fluorescent cationic cyanine dye largely used in membrane transport studies and to measure P-gp activity. R123 accumulates within the cell in electrically negative compartments like mitochondria, and therefore it is referred as a mitochondria-specific dye that does not bind to DNA. In addition, R123 has a great affinity to P-gp, therefore it is an appropriate substrate for such studies. R123 has a maximum absorption at 485nm and a maximum emission at 530nm (Johnson et *al.*, 1981).

Fluorescein: Fluorescein (FL) is a fluorescent non-toxic anionic dye used to measure MRP1 functional activity (Huai-Yun et *al.*, 1998). FL is accumulated in the cytoplasm of the cell. FL is a well-recognized substrate of MRP1, and therefore it can easily be used for such studies. FL has a maximum absorption at 490nm and maximum emission at 520nm.

Doxorubicin: Doxorubicin (Dox) is a cytotoxic anthracycline antibiotic isolated from the fungus *Streptomyces peucetius,* and is widely used in chemotherapy to treat various cancers (Dorr et *al.,* 1994). Dox inhibits DNA topoisomerase II activity, an essential enzyme responsible for uncoiling and repairing damaged DNA. Dox binds non-covalently to DNA and damages DNA by intercalation of the anthracycline segment, metal ion chelation, or by the formation of free radicals (de Beer et *al.*, 2001). Resistance to Dox could be caused by the presence of P-gp, MRP1 and BCRP protecting the cell. Hence Dox is a know substrate of these efflux proteins. However, mechanisms other than active efflux

mediated by ABC proteins, such as the decrease of the activity of topoisomerase II and increase of the GSH detoxification system could also be involved in the drug resistance. Dox shows strong red fluorescence and therefore its accumulation in cells can be easily measured. Dox has a maximum absorption at 488nm and maximum emission at 520nm. The intensity of fluorescence is decreased when Dox binds to DNA. Therefore prior to measurement, Dox accumulated in cells needs to be released by lysis of the cells with 1% sodium dodecyl sulphate (SDS).

Topotecan: Topotecan (TPT) is an anticancer drug, a semi-synthetic derivative of camptothecin that inhibits topoisomerase I activity. It is widely used in chemotherapy to treat ovarian and certain types of lung cancer. TPT binds to topoisomerase I-DNA complex and prevents DNA single strand religation. TPT exhibits its cytotoxicity when DNA replication enzymes interact with the ternary complex formed by TPT during DNA synthesis, therefore causing DNA damage (Nagourney et *al.*, 2003). However, the therapeutic efficacy of TPT is reduced by its hydrolysis. The biological activity to inhibit topoisomerase I is specific to TPT in its lactone form, which is stable in acidic pH (Kollmannsberger et *al.*, 1999). TPT is a known substrate of BCRP and shows yellow fluorescence. It has maximum excitation wavelength at 360nm and a maximum emission wavelength at 530nm.

Mitoxantrone: Mitoxantrone (MTX) is a synthetic anticancer anthracenedione antibiotic, mainly used to treat breast cancer, myeloid leukaemia and non-Hodgkin's lymphoma. MTX intercalates the DNA and inhibits topoisomerase II activity that is essential for DNA synthesis and repair. MTX has a dark blue colour and has two major absorption peaks at the wavelengths 610nm and 660 nm (Koeller and Eble, 1988; Hagemeister et *al.*, 2005). MTX is a well-known substrate of P-gp and BCRP.

17-ß-Estradiol: 17-ß-Estradiol (E2) is a potent human estrogenic hormone produced in the ovaries, testes, placenta, and adipose tissue. 17-ß-Estradiol may be glucuronidated or sulfated via interohepatic recirculation. It is known to be a substrate of MRP1 and BCRP. However, a new study has demonstrated E2 to be an inhibitor of BCRP, increasing the

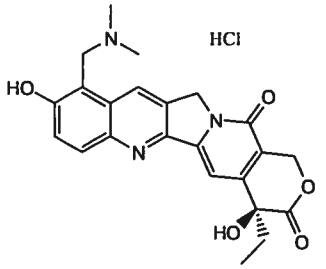
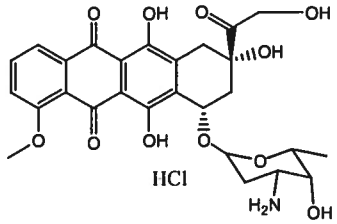
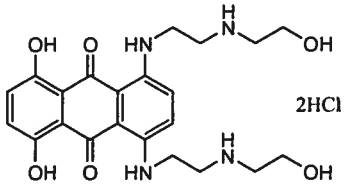
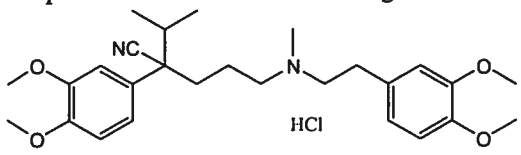
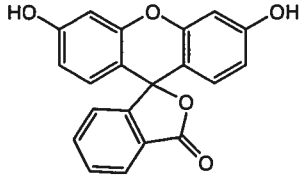
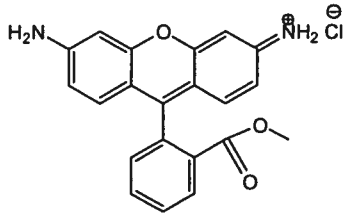
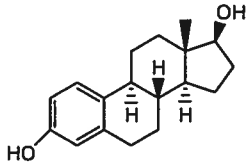
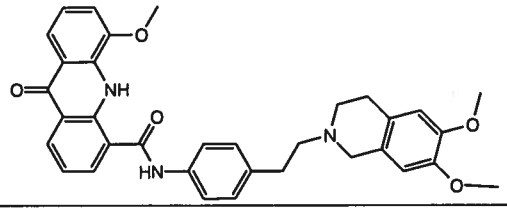
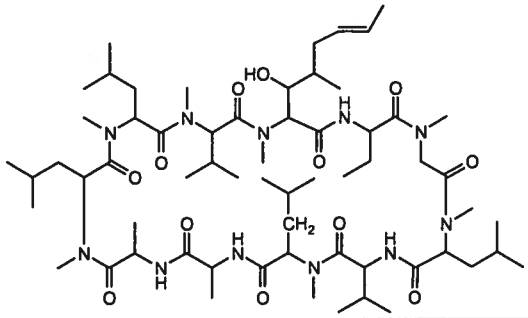
accumulation of topotecan in MCF7/BCRP cells overexpressing BCRP (Imai et *al.*, 2005). Therefore, we also included E2 as a potential BCRP inhibitor.

Verapamil: Verapamil (VP) is a calcium channel blocker that blocks the transport of calcium into smooth muscle cells, hence relaxing and dilating the arteries (McTavish et *al.*, 1989). VP is one of the first identified inhibitors of P-gp, by blocking P-gp modulated efflux and increases the accumulation of various anti-cancer drugs. VP was demonstrated to be a competitive inhibitor; however, it requires elevated concentrations in plasma to achieve an inhibitory effect *in vivo*. Such concentrations of VP may be toxic. Previous studies have shown that VP exhibits an effective inhibitory activity on MCF-7 ADR cells overexpressing P-gp (communication by Supratek Pharma).

Cyclosporin A: Cyclosporin A (CSA) is an immunosuppressant drug produced by the fungus *Hypocladium inflatum gams* initially isolated from Norwegian soil. CSA is mainly used after organ transplant surgery to reduce the immune system response to reject the transplant. CSA binds to lymphocytes and reduces the function of effector T-cells (Gao et *al.*, 1989; Goldfeld et *al.*, 1994). CSA is known as a competitive inhibitor of P-gp. Previous studies have shown that CSA exhibits an effective inhibitory activity on MCF-7 ADR cells overexpressing P-gp. Therefore, CSA is included as a P-gp inhibitor in this study (communication by Supratek Pharma).

GF120918: Also know as Elacridar or GG918 is a synthetic non-specific potent inhibitor of P-gp and BCRP discovered and developed by GlaxoSmithKline. GF120918 is an acridone carboxamide derivative that has been demonstrated to reverse MDR mediated by P-gp and BCRP (de Bruin et *al.*, 1999; Kruijtzer et *al.*, 2002).

Table 1.8 Cytotoxic drugs and diagnostic probes used in this study

Topotecan Molecular Weight: 457.91 	Doxorubicin Molecular Weight: 543.52 
Mitoxantrone Molecular Weight: 517.40 	Verapamil Molecular Weight: 491.06 
Fluorescein Molecular Weight: 332.31 	Rhodamine123 Molecular Weight: 380.82 
17 β-estradiol Molecular Weight: 272.38 	GF120918 Molecular Weight: 563.64 
Cyclosporin A Molecular Weight: 1202.61 	

1.8.3 Accumulation studies

The intracellular drug/probe accumulation studies can be used to screen substrates and inhibitors of ATP-dependent efflux pumps. One common method of analysis of cellular accumulation of fluorescent probes is flow cytometry, a powerful technique used to measure fluorescence in individual cells. It allows one to estimate the viability of the cells in population and it quantifies the fluorescent accumulation within individual cells rather than merely average values for a population. Hence, flow cytometry is highly sensitive. However, this method is rather expensive, and it is not suitable for screening samples by high-throughput.

Measuring cell-associated fluorescence through a fluorescent plate reader (FPR) has been widely used as an effective alternative method to detect fluorescent probes in cells (Batrakova et *al.*, 2003). FPR is conducive to high-throughput screening. The plate readers are either filter-based or monochromator-based. Microplate readers can measure either 96, 384 or 1536-well plates. In the filter-based FPR, the fluorescent reading applies light (for example from tungsten halogen), which first passes by an excitation filter, then excites the fluorescent sample in a plate well. The light emitted from the plate passes by an emission filter and finally reaching a photomultiplier detector for measurement. Most fluorescent readers have top and bottom read capabilities. Many FPR are equipped with several measurement functions. Besides fluorescence intensity they may also measure absorbance, monitor temperature of a sample, measure time-resolved fluorescence, fluorescence polarization and luminescence. FPR advantages include rapid analysis, low-cost, and analysis of large number of samples at once. However, compared to flow cytometry, major disadvantages of FPR are: relatively low sensitivity, large errors resulting from the averaging of fluorescence measured on multiple cells at once, and lack of ability to detect the viability of measured cells.

Previous studies by Supratek Pharma and Venne et *al.*, on the inhibitory effect of Pluronics® on P-gp mediated drug efflux were evaluated by flow cytometry. These studies showed a promising inhibitory effect with Pluronics® on cells overexpressing P-gp (Venne et *al.*, 1996; personal communication by Supratek Pharma). However, for the present studies, due to the planned large number of samples, we decided to employ a

fluorescent/absorbance plate reader, which is an effective instrument that is widely used for faster screening.

1.8.4 Cytotoxicity studies

To examine the effect of Pluronics® on the cytotoxicity of drugs, the cells were exposed to Dox or TPT, and eventually to inhibitors of the efflux systems. Pluronic® L61, a highly active poloxamer inhibiting P-gp mediated efflux, was used at a concentration 0.05%, which is its most effective concentration. The cytotoxicity was determined using the standard XTT assay (Scudiero, 1988). XTT (2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H tetrazolium-5-carboxanilide sodium salt) is metabolically reduced in viable cells to water-soluble farmozan product, which can be directly assessed by absorbance readings. Further, the sensitivity of the XTT method can be improved by the addition of phenazine methosulfate (PMS) that enhances the cellular reduction of XTT. After treatment of the cells with a cytotoxic drug at various concentrations and with or without the presence of inhibitors, the IC50 corresponding to 50% of the cell proliferation can be determined. The biological effect of various inhibitors can be evaluated by the comparison of the IC_{50} values obtained in their presence.

1.9 Hypothesis

In this study we propose the following two hypotheses:

1) Polymers of the Pluronic® group inhibit MRP1 and BCRP-mediated drug efflux.
2) Pluronics® are as effective as other known inhibitors which chemosensitize P-gp, MRP1 and BCRP expressing cell lines.

1.10 Objectives

1.10.1 General objective

The main objective of the present study was to study *in vitro* the effects of Pluronic® block copolymers on the transport function of three ABC proteins implicated in drug resistance: P-gp, BCRP and MRP1.

1.10.2 Specific objectives

1) To evaluate the critical micelle concentration (CMC), one of the basic surfactant properties of Pluronics®.

For this purpose, twenty-one Pluronics® were tested and CMC is determined by measuring pyrene fluorescence.

2) To test *in vitro* the inhibitory and sensitizing activity of Pluronics® on Pgp, BCRP and MRP1 overexpressing cells.

For this purpose, absorption and cytotoxicity studies are performed on breast cancer cell line, MCF-7 ADR (doxorubicin-resistant) which overexpresses P-gp, MCF-7 TPT (topotecan-resistant) which overexpresses BCRP, and the leukemia cancer cell line HL-60 ADR (doxorubicin- resistant) which overexpresses MRP1. MCF-7 TPT cells were derived by stepwise selection from parental MCF-7 cells exposed to topotecan (TPT) hydrochloride. The accumulation studies were performed in the presence of:

- *Polymers: twenty-one Pluronics® were tested on P-gp overexpressing cells then Pluronics® L61 and P85 were selected for further studies*
- *Probes: R123, fluorescein*
- *Anti-cancer drugs: doxorubicin , mitoxantrone, topotecan*
- *MDR inhibitors: cyclosporin A, verapamil, 17 ß-estradiol and, GF120918 for comparison to the inhibitory potency of Pluronics®*

The sensitizing activity of Pluronics® was tested to the cytotoxic drugs doxorubicin and topotecan by Pluronic® L61. The viability of cells was examined using standard XTT assay.

3) To examine substrate selectivity of P-gp, BCRP and MRP1 efflux systems exposed to Pluronics®.

CHAPTER 2: Materials & Methods

2.1 Measurement of critical micelle concentration (CMC) of Pluronics®

Each tested Pluronic® (BASF, New Jersey, USA) was dissolved in phosphate buffer solution (PBS) pH 7.2 (Invitrogen, Grand Island, NY, USA) to form a stock solution. The concentration of Pluronic® has been selected such that the stock solution was transparent by visual inspection at 37 °C. Each stock solution was then diluted with PBS to obtain a series of 32 working solutions of various concentrations covering four orders of magnitude. The solutions were incubated at room temperature for one hour. Pyrene, 99% optical grade, (Aldrich, Milwaukee, WI, USA) was dissolved in acetone, HPLC grade, (Laboratoire Mat, Beauport, QC, Canada) to form a 5μM solution. 30μl of this solution was transferred into each well of a Hellma quartz 96- well plate (Bio-tek, Vermont, USA). The plate was left uncovered at room temperature until the acetone evaporated, leaving pyrene deposit. 300μl of Pluronic® solution was then placed into each well. The plate was covered with Parafilm (Pechiney Plastic Packaging, WI, USA) and incubated at 37° C for 4 hours. The fluorescence of the samples on the plate was measured with a FL 600 Micro Plate Fluorescence Reader (Bio-Tek Instruments, Winooski, VT, USA). The data obtained for each polymer were represented in a graph of concentration of polymer versus fluorescence intensity. Each graph has an "S"shape with a plateau for the lowest and the highest concentrations of the measured range, or the graph represents a part of this "S"shaped curve. CMC was estimated from the graphs. The lowest concentration at which an increase of fluorescence from plateau was noted and determined to be the CMC (refer to figure 3.1, page 70 and also reference: Kabanov et *al.*, 1995).

2.2 Cell culture

2.2.1 Cell lines

Human breast carcinoma MCF-7 (sensitive parental cells) were purchased from (American Type Culture Collection, VA, USA). The MCF-7 ADR (doxorubicin resistant) cell subline derived from parental MCF-7 cells by culturing in the presence of doxorubicin (Dox) as kindly supplied by Dr. YL. Lee (William Beaumont Hospital, Royal Oak. MI, USA) (Batist *et al*, 1986). The MCF-7 TPT (topotecan resistant) cell subline was derived by stepwise selection from parental MCF-7 cells exposed to topotecan (TPT) hydrochloride. MCF-7 cells were cultured in Dulbecco's Eagle's medium (D-MEM) containing 10% heat inactivated fetal bovine serum, 1% HEPES buffer solution and 1% penicillin -streptomycin-amphotericin B (all from Invitrogen, Grand Island, NY, USA). After the cells proliferated actively, they were exposed to increasing concentrations of TPT hydrochloride (Hande Tech Development Co. Inc.,TX, USA), ranging from 50, 100, 150, 200 and 240nM. After six months and 120 passages, MCF-7 cells resistant to 240nM TPT were obtained. The expression of *BCRP and P-gp* genes in MCF-7 TPT cells was analyzed by Dr. A. Semov using quantitative reverse transcription-polymerase chain reaction (RT-PCR); a 16-fold increase of *BCRP* expression and 3.6-fold increase of *P-gp* expression relative to MCF-7 topotecan sensitive parental cells normalized to 1-fold for both genes was determined (communication by A. Semov, Supratek Pharma). Human leukemia cancer cell lines, parental HL 60 sensitive and HL 60 ADR (doxorubicin resistant) were kindly supplied by Dr. Alexander V. Kabanov (University of Nebraska Medical Center, Nebraska, USA).

2.2.2 Cell culture conditions

Adhesive cells; MCF-7, MCF-7 ADR and MCF7-TPT were cultured in D-MEM medium. Suspended cells HL 60 and HL 60 ADR cells were cultured in RPMI 1640 medium (Invitrogen, Grand Island, NY, USA). Both media were complemented with 10% heat inactivated foetal bovine serum, 1% HEPES buffer solution and 1% penicillin - streptomycin-amphotericin B. Cells were cultured in 25 cm^2, 75 cm^2 or 175 cm^2 polystyrene culture flasks (Sarstedt, Montreal, Quebec, Canada). The medium for culturing

MCF-7 ADR cells was complemented with 1μg/ml Dox hydrochloride (Yunnan Hande Tech Development Co Ldt, Kunming, China). The medium for culturing MCF-7 TPT cells was complemented with 240nM TPT hydrochloride (Hande Tech Development Co. Inc.,TX, USA) initially prepared in sterile Mili-Q water supplemented with 0.1% citric acid (Aldrich, Milwaukee, WI, USA). Cells were kept in an incubator (Revco technologies, Asheville, NC, USA) at continuous logarithmic growth at 37° C in a humidified atmosphere with 5% CO_2. After reaching 80-90% confluency (typically within 4-5 days, for MCF-7 TPT 1-2 weeks), adhesive cells were detached through by standard trypsinisation (Freshney, 1988) with 1% trypsin-EDTA (Invitrogen, Grand Island, NY, USA). Cells then were washed with (PBS) pH 7.2 and recultured in media. Cells were seeded at a density of 12,000- 20,000 cells/cm^2 into a new cell flask.

2.3 Drug/probe accumulation studies

Confluent cell monolayers were washed, trypsinized with trypsin-EDTA, and washed once with PBS at pH 7.2. All cells after washing were distributed into 5 ml polystyrene tubes (Fisher, Hampton, NH, USA) $5x10^5$ cells (500ul) per tube. The tubes were centrifuged at 1200RPM (Beckman Coulter GS-6R centrifuge, Fullerton, CA, USA) and the PBS was decanted. The cells were then resuspended in solutions containing various diagnostic probes and / or inhibitors in media; the tested probes included Rhodamine 123 (Sigma, St-Louis, MO, USA), fluorescein (Aldrich, St-Louis, MO, USA) and anti-cancer drugs: Dox hydrochloride, TPT hydrochloride, MTX hydrochloride (Sigma, St-Louis, MO, USA); the inhibitors included Pluronics®, 17β-estradiol (Sigma, St-Louis, MO, USA), verapamil hydrochloride (Sigma, St-Louis, MO, USA), cyclosporin A (Sigma, St-Louis, MO, USA) and GF120918 was kindly supplied by GlaxoSmithKline (Hertfordshire , United Kingdom). Cells were incubated at 37° C with 5% CO_2 for 45 minutes. After the incubation, cells were centrifuged at 1200 RPM washed once with 4 ml of cold PBS, and resuspended in 500 μl cold PBS. Cell suspensions, 300 μl/well, were transferred into 96-well polystyrene plates (Sarstedt, Montreal, Quebec, Canada), and fluorescence was measured using a Microplate Fluorescence Reader FL 600 (BioTek, Vermont). For the experiments with Dox, 1% sodium dodecyl sulphate (SDS) was added

into each well and incubated for 15 minutes on ice before taking a reading. The wavelength settings for measurement were as follows: for R123 and for FL λ_{Ex} 485nm, and λ_{Em} 530nm; for TPT λ_{Ex} 360nm and λ_{Em} 530nm; for Dox λ_{Ex} 485nm and λ_{Em} 590. Accumulation of MTX was determined by measurement of optical density of the cell suspension at the wavelength 660nm using a Spectra Max Plus UV plate reader (Molecular devices, CA, USA).

2.4 Cell-based cytotoxicity assay

2.4.1 Drug-polymer treatment

The cells were distributed into 96-well polystyrene plates (Sarstedt, Montreal, Quebec, Canada), 3500 cells/well for MCF-7 and MCF-7 ADR; 8000 cells/well for MCF-7 TPT; 10 000 cells/well for HL 60 and HL 60ADR, and incubated overnight in a humidified incubator at 37° C and 5% CO_2.

Aqueous stock solutions of doxorubicine hydrochloride (Dox) 0.8 mg/ml, or topotecan hydrochloride (TPT) 0.8 mg/ml were added to D-MEM complete medium with the pH adjusted to 5.5 with HCl to obtain the following solutions of cytotoxic drugs: 0.05 mg/ml Dox for MCF-7; 0.25 mg/ml Dox for MCF-7 ADR; 0.01mg/ml Dox for HL 60; 0.1 mg/ml Dox for HL 60 ADR; 0.025 mg/ml TPT for MCF-7; 0.25 mg/ml TPT for MCF-7 TPT. Similar solutions of cytotoxic drugs were also prepared and supplemented with 0.05% Pluronic® L61.

These solutions were further serially diluted five-fold with the same respective media. 200 µl of each solution were added to cells in a well, in triplicates, and the plate was incubated for 2h in a humidified incubator at 37° C and 5% CO_2. Then cells were washed once with their respective 200 µl complemented media and they were allowed to grow for 3 days in fresh complete culture media. Viability of cells was then determined using a standard XTT assay (Scudiero 1988).

2.4.2 Treatment with XTT

2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H tetrazolium-5-carboxanilide sodium salt (XTT) and phenazine methosulfate (PMS), both from (Sigma, MO, USA) were dissolved in PBS pH 7.2 (1mg/ml XTT and 1.54mg/ml PMS). 1ml of XTT solution and 5µl of PMS were mixed and further diluted four times with PBS pH 7.2. Media were discarded from the cells, and 200µl of the mixed solution of XTT and PMS was added to each well. The plate was incubated in a humidified incubator at 37° C and 5% CO_2 for 2 hours). The optical density of the samples on plate was measured at 450nm with a Spectra Max Plus UV plate reader (Molecular devices, CA, USA). The results are the means of these samples and standard deviations are represented in graphs. The highest OD measured at low concentrations of the cytotoxic drug represented to uninhibited cell growth, and the lowest OD measured at high concentration of the drugs represented maximum (100%) inhibition of cell growth. These OD measurements were used to normalize the results for other samples in the series. IC_{50} (concentration of Dox or TPT corresponding to 50% growth inhibition) was estimated from the graphs.

2.5 Statistical analysis of the results of accumulation and cytotoxicity assays

All the experiments were performed in triplicate. The data represent means of three experiments, and error bars represent standard deviations. The significance of results was evaluated by Student's *t*-Test, assuming two-tailed distribution of samples with equal variance. The results were considered significantly different if $P \leq 0.05$.

CHAPTER 3: Results

3.1 Critical micelle concentration of polymers (CMC)

The critical micelle concentration (CMC) of a polymer is the lowest concentration at which unimers aggregate and self-assemble into micelles. In order to understand the aggregation state of Pluronics® in the following cell-based experiments, their CMC has been determined. This has been done by measuring pyrene fluorescence. Pyrene is a hydrophobic fluorescent compound and its fluorescence increases when pyrene is dissolved in a hydrophobic environment such as in micelle cores. Consequently, the fluorescence of pyrene increases with micelle formation.

The fluorescence of pyrene was determined in the presence of Pluronics® at concentrations between 0.0001% and 1%, at 37°C, at pH 7.2. The fluorescence was measured with a fluorescence plate reader (λ_{Ex}=340nm and λ_{Em}=400nm). Twenty-one Pluronics® were tested. Figure 3.1 presents an example of fluorescence data measured for the samples containing Pluronic® P85. The minimum concentration of the polymer at which the fluorescence intensity begins to rise is 0.03% (Figure 3.1). This concentration corresponds to CMC of Pluronic® P85. CMC values of all tested Pluronics® are presented in Table 3.1; CMC values ranged from 0.0007% to 0.4%.

Due to the equilibrium nature of polymer aggregation it is not possible to precisely determine the concentration at which the transition from unimers to micelle formation starts and at which concentration it ends. Consequently CMC is not a precisely defined value. The accuracy of the CMC values is such that the error of log(CMC) is ±0.5 or less.

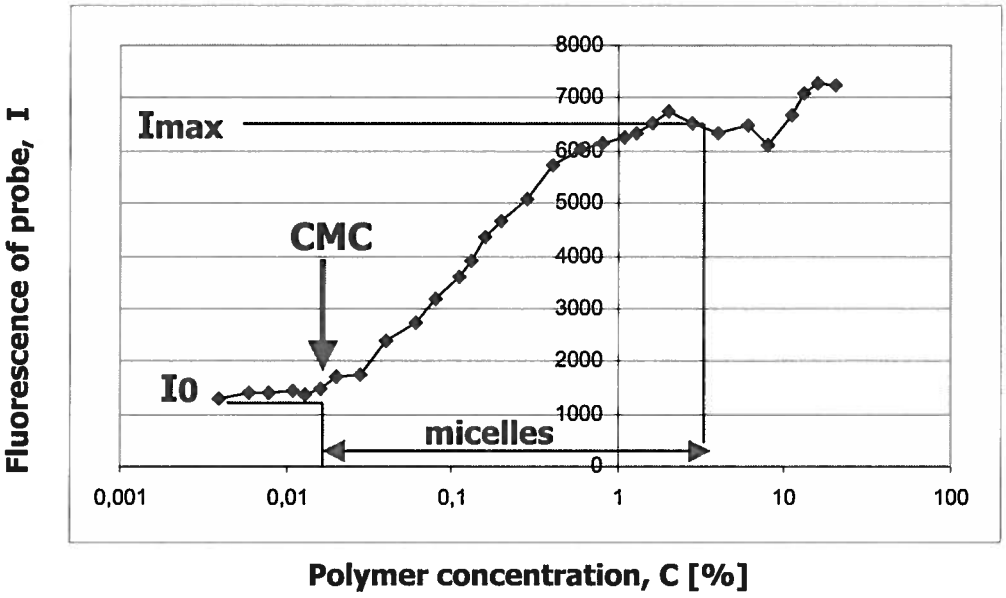


Figure 3.1 Critical micelle concentration (CMC) of Pluronic® P85.
The graph represents the increase of pyrene fluorescence in the presence of Pluronic® P85 at increasing concentrations. Fluorescence was measured with a fluorescence plate reader (λ_{Ex}=340nm and λ_{Em}=400nm). The initial intensity (I_0) represents fluorescence in diluted polymer solution where no aggregates are observed. The maximum intensity (I_{max}) is related to pyrene totally incorporated into polymer aggregates in colloid solution. The lowest concentration at which the fluorescence is notably raised from low plateau relates to CMC, critical micelle concentration, the lowest concentration at which the polymer molecules aggregates. For Pluronic® P85 the CMC is 0.03% (Kabanov et *al.*, 1995).

3.2 Intracellular accumulation studies

3.2.1 Preliminary accumulation studies on cells expressing the P-gp transporter

To evaluate the effect of Pluronics® on the functional activity of P-gp in cells, the accumulation of R123, a substrate of P-gp was tested on the MDR resistant cells MCF-7 ADR (expressing P-gp transporter) in the presence of 21 different Pluronic® compounds. The cells were incubated with R123 (0.5μM) and with each of the Pluronics® at the following concentrations 0.005%, 0.02%, 0.05%, 0.2%, 0.5 %, 2%, at 37°C for 45 minutes. Fluorescence of the cells after incubation was measured by fluorescence plate reader. Several Pluronics® increased the intracellular accumulation of R123 in MCF-7 ADR cells, and this effect was concentration dependent with a bell-shaped curve. Full results are not presented herein; the examples of data for Pluronic® L61 and Pluronic® P85 are shown in Figure 3.3.

The effective concentration of Pluronics® upon which R123 accumulation reaches the maximum level, and the relative enhancement of R123 accumulation are presented in Table 3.1. The enhancement of R123 accumulation by various Pluronics® has been normalized by the comparison to a standard sample containing 0.05% Pluronic® L61. The maximum effective concentration of Pluronics® ranged from 0.02% to 0.5%, and the relative enhancement of R123 accumulation ranged from 0 to 1.3, compared to the effect of 0.05% Pluronic® L61. Pluronics® showed no effect on accumulation of R123 in parental drug-sensitive MCF-7 cells (communication by Supratek Pharma, results not presented herein).

Based on the presented results, and on physicochemical characteristics of the polymer (HLB, molecular weight, CMC), and also considering the previously published information about chemosensitizing effect of Pluronics® on several MDR cell lines (references, Batrakova et *al.*, 1998 and 2001a), a diversified set of polymers has been selected for further studies: Pluronic® L61, P85, L92, P123 and F127. The concentrations of selected polymers used in further studies were selected to cover the range below and above the respective CMC, and included the maximum effective concentrations listed in Table 3.1.

Table 3.1 Critical micelle concentration (CMC) and the enhancement of intracellular rhodamine (R123) accumulation by Pluronic® polymers.

The critical micelle concentration (CMC) for each polymer was determined by measuring the fluorescence of pyrene (λ_{Ex}=340nm and λ_{Em}=400nm) in the presence of various polymer concentrations. R123 (0.5μM) accumulations in adriamycine resistant cells MCF-7 ADR (expressing P-gp transporter) were measured by R123 fluorescence (λ_{Ex}=485nm and λ_{Em}=530nm) after 45-minute incubation in the presence or absence of polymers.

BASF polymers Pluronic®	CMC [a] [%]	Maximum effective concentration of polymer [b] [%]	Relative enhancement of R123 accumulation[c] (enhancement by 0.05% Pluronic® L61 equals 1.00)
L10	0.03	0.2	0.91 ± 0.01
L31	0.3	0.5	0.66 ± 0.04
L35	0.4	nt	nt
F38	0.4	0.05	0.00 ± 0.05
L43	0.3	0.05	0.16
L44	0.4	0.05	0.00 ± 0.05
L61	0.06	0.2	1.00 ± 0.02
L64	0.2	0.2	0.91 ± 0.01
F68	0.3	nt	nt
L81	0.03	0.02	1.2
P84	0.02	0.2	0.92 ± 0.09
P85	0.03	0.2	0.56 ± 0.07
F87	0.2	nt	nt
F88	0.4	nt	nt
L92	0.003	0.02	1.25 ± 0.17
F108	0.02	nt	nt
L101	0.0007	0.05	1.09 ± 0.06
P104	0.002	0.5	0.69 ± 0.04
L121	0.002	0.2	0.95 ± 0.14
P123	0.002	0.5	1.12 ± 0.07
F127	0.003	0.05	0.01 ± 0.05

[a] Average of three measurements; the error of log(CMC) is ±0.5 or less

[b] The maximum effective concentration was selected from the series of following tested concentrations: 0.005, 0.02, 0.05, 0.2, 0.5, 2 [%]

[c] The R123 accumulation enhancement caused by polymers at their respective maximum effectiveness with standard deviation, compared to the effect of 0.05% Pluronic® L61.

nt: not tested (Note: Batrakova et *al.*, 2003 have shown the inactivity of hydrophilic Pluronics® in sensitizing P-gp efflux systems, therefore these polymers were exempt from our studies for further testings).

3.2.2 *Effect of Pluronics® on cells expressing the P-gp transporter*

The effect of previously selected Pluronic® block copolymers on P-gp functional activity was evaluated using the same method as described in the previous chapter, the preliminary R123 accumulation studies. The accumulation of R123 (0.5μM) in the presence of different Pluronics® (L61, P85, L92 or P123) at the respective maximum effective concentration is presented in Figure 3.2. The values were normalized by comparison with the effect of a standard 0.05% solution of Pluronic® L61. All the selected polymers increased the accumulation of R123 in MCF-7 ADR cancer cells, and the increase varied from 0.7 to 1.2, relative to the effect of 0.05% L61. Pluronic® P123 at a concentration of 0.2% and Pluronic® L92 at 0.02% were equal to or more effective than Pluronic® L61 at 0.05% (respectively, 1.1 and 1.2 effect of Pluronic® L61). Pluronic® P85 at 0.2% was less effective, showing an activity of 0.6 compared to Pluronic® L61.

The effect of Pluronics® L61 and P85 on R123 accumulation in MCF-7 ADR cells was concentration-dependent with a bell-shaped curve (Figure 3.3). The most effective concentration of Pluronic® L61 was 0.05% which corresponded well to the CMC of this polymer (0.06%; Table 3.1). The absolute increase of accumulation of the fluorescent probe in the tested system was 14-fold. In the case of Pluronic® P85 the maximum effective concentration 0.2%, was higher than the CMC of this polymer (0.03%; Table 3.1). This concentration of Pluronic® P85 caused a significant 5.7-fold absolute increase of R123 accumulation (P=0.0001).

The studies of uptake of the anti-cancer drug Dox into MCF-7ADR cells in the presence of Pluronics® L61 and P85 showed increased accumulation of this drug in cells compared to cells lacking exposure to polymers (control). The accumulation was concentration-dependent (Figure 3.4). Pluronic® L61 at 0.05% and Pluronic® P85 at 0.2% enhanced the accumulation of Dox (20μM) in cells respectively by 2.4-fold and 2.3-fold, which was lower than the respective effect on accumulation of R123. Higher concentrations of Pluronics® occasionally caused increased accumulation of Dox; we assume that this effect is caused by interaction of polymer aggregates with the cells. Pluronics® showed no effect on the accumulation of Dox in parental drug-sensitive MCF-7 cells (communication by Supratek Pharma, results not presented in this study).

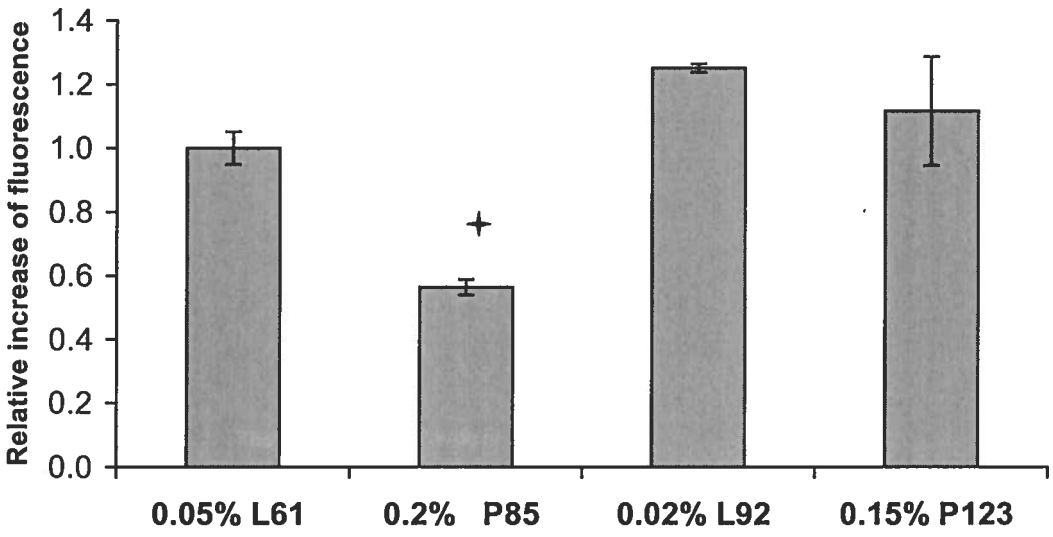


Figure 3.2 Enhancement of R123 uptake to MCF-7 ADR cells in the presence of Pluronics® P85, L92 or P123, relative to the effect of Pluronic® L61 at concentration 0.05%.

The modulatory effect of Pluronics® P85, L92 and P123 on accumulation of R123 (0.5μM) in MDR resistant cells MCF-7 ADR (expressing P-gp) was studied by the measurement of R123 fluorescence (λ_{Ex}=485nm and λ_{Em}=530nm) with a plate reader. The presented concentration for each polymer corresponds to their maximum effective activity. The experiments were repeated in triplicate. Pluronic® P123 and Pluronic® L92 were equally or slightly more potent than Pluronic® L61 at their most effective concentrations.

+ Denotes results that are significantly different from Pluronic® L61 at 0.05% ($P \leq 0.05$).

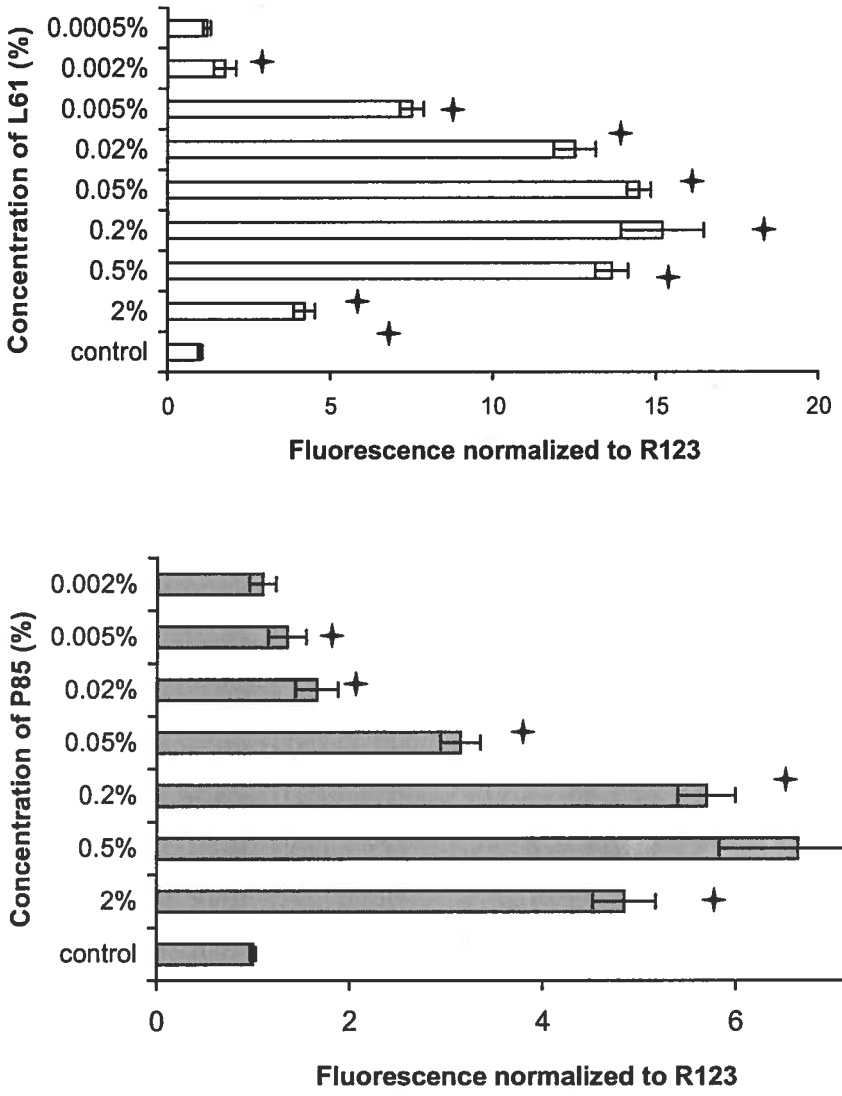


Figure 3.3 Concentration-dependent effect of Pluronic® L61 or Pluronic® P85 on the accumulation of rhodamine (R123) in MCF-7 ADR cells.

The modulatory effect of Pluronics® L61 and P85 on the accumulation of R123 (0.5μM) in MDR resistant cells MCF-7 ADR (expressing P-gp) was studied by the measurement of R123 fluorescence (λ_{Ex}=485nm and λ_{Em}=530nm) with a plate reader. The range of tested concentrations of each polymer included the "control" (without the presence of polymer). The experiments were repeated in triplicate and the fluorescence was normalized to R123 alone (control =1). The effect of Pluronics® L61 and P85 on MCF-7 ADR cells was concentration-dependent with a bell-shaped curve. Pluronic® L61 demonstrated higher sensitizing activity then Pluronic® P85.

+ Denotes results that are significantly different from R123 alone (control) ($P \leq 0.05$).

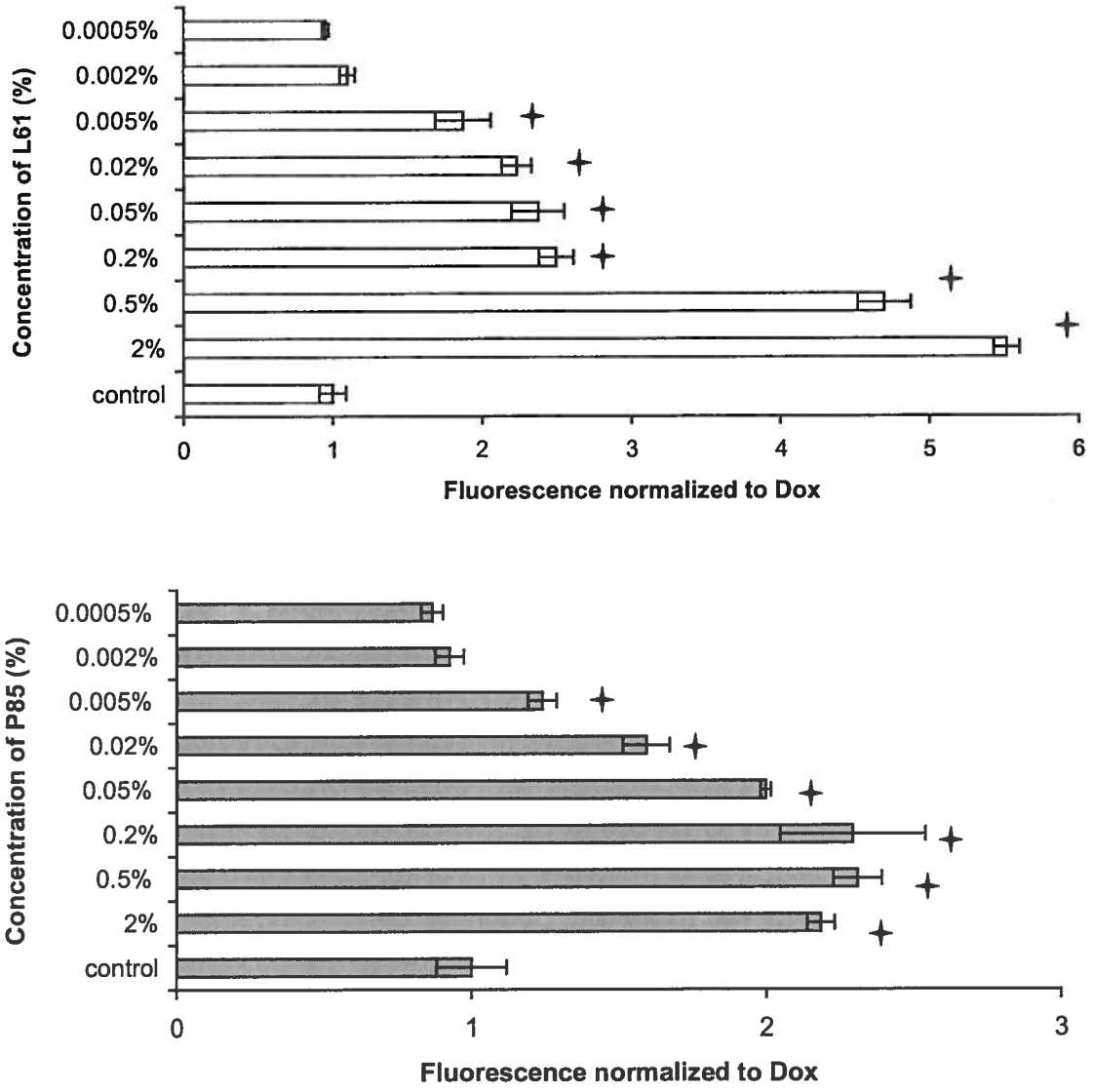


Figure 3.4 Accumulation of doxorubicin (Dox) in MCF-7 ADR cells in the presence of Pluronic® L61 or Pluronic® P85 at various concentrations.

The modulatory effect of Pluronics® L61 and P85 on the accumulation of Dox (20μM) in MDR resistant cells MCF-7 ADR (expressing P-gp) was studied by the measurement of Dox fluorescence (λ_{Ex}=485nm and λ_{Em}=590nm) with a plate reader. The range of tested concentrations of each polymer included the "control" (without the presence of polymer). The experiments were repeated in triplicate and the fluorescence was normalized to Dox alone (control =1). The effect of Pluronics® L61 and P85 on MCF-7 ADR cells was concentration-dependent with a bell-shaped curve for P85. Pluronic® L61 demonstrated higher sensitizing activity then P85.

+ Denotes results that are significantly different from Dox alone (control) ($P \leq 0.05$).

3.2.3 *Effect of Pluronics® on cells expressing BCRP and P-gp transporters*

To evaluate the effect of Pluronics® on the functional activity of BCRP in cells, the accumulation of the anti-cancer drug topotecan hydrochloride (TPT), a substrate of BCRP was tested in the presence of several Pluronics®. TPT is chemically unstable at neutral and basic pH because its lactone ring is being hydrolyzed to acid alcohol. The lactone form is the one responsible for inhibition of topoisomerase I, and therefore the biological activity of TPT and its therapeutic efficacy in solution at neutral pH is quickly reduced by hydrolysis. Therefore the conditions of accumulation experiments were tested at a range of pH levels to asses the effect of pH on Pluronic® activity. Initially we tested R123 accumulation in MCF-7 ADR cells at following pH: 4.5, 5.5, 6.5, 7.5, 8.5, in the presence or in absence of 0.05% Pluronic® L61. The results presented in Figure 3.5 demonstrate that the change of pH in the range 5.5-8.5 does not significantly affect R123 accumulation in the tested cells, and the effect of Pluronic® L61 on accumulation is only slightly affected by the pH change. Hence, further studies on the accumulation of TPT were performed at pH 5.5.

The modulatory effect of Pluronics® on the accumulation of TPT in MCF-7 TPT cells (expressing BCRP transporter) was tested by exposing the cells to TPT, (50μM) in the presence of either Pluronic® L61 (0.05%) or P85 (0.2%). For the comparison, the same effect was also tested on sensitive cells MCF-7, and on MCF-7 ADR cells (expressing P-gp transporter). In addition, the inhibitory effect of polymers was compared to that of a non-specific BCRP and P-gp inhibitor GF120918 (10μM). Results demonstrated a higher increase for the accumulation of TPT in MCF-7 ADR and MCF-7 TPT cells in the presence of GF120918 compared to cells exposed to the polymers (Figure 3.6). GF120918 increased the TPT uptake 2.4-fold in MCF-7 ADR cells and 1.5-fold in MCF-7 TPT cells. Pluronics® L61 and P85 enhanced TPT uptake only in MCF-7 ADR cells (1.8-fold and 1.7-fold, respectively). Neither polymer altered the accumulation of the drug in MCF-7 TPT cells. In the parent MCF-7 cells, the polymers did not affect the accumulation of TPT whereas GF120918 caused a small increase (1.3-fold).

For comparison, the accumulation of R123 (0.5μM) in the same three cell lines: MCF-7 sensitive, MCF-7 ADR and MCF-7 TPT were tested. The experiment was performed in the exact same conditions as the accumulation experiments involving TPT:

pH 5.5, presence of Pluronic® L61, Pluronic® P85 or GF120918. In MCF-7 ADR and MCF-7 TPT cells both polymers enhanced the accumulation of R123 more than GF120918 did (Figure 3.7). An increase of R123 accumulation was also detected in MCF-7 sensitive cells when exposed to Pluronic® L61 and Pluronic® P85 (2.6-fold and 1.4-fold, respectively). MCF-7 TPT cells showed no increase of accumulation of R123 in the presence of GF120918. However, the accumulation of R123 in MCF-7 TPT cells was increased 2.7-fold in the presence of Pluronic® L61 and increased 2.2-fold in the presence of Pluronic® P85. Consistently with the Pluronic® effect described in the previous studies on the uptake of R123 in MCF-7 ADR cells (Figure 3.3), Pluronic® L61 caused up to a 4-fold increase of R123 accumulation in MCF-7 ADR cells and a lower R123 accumulation (2.4-fold increase) was observed when the same cells were exposed to Pluronic® P85 (Figure 3.7).

To further study the effect of inhibitors and their specificity on transporters, the accumulation of TPT (50μM) and R123 (0.5μM) in MCF-7 TPT cells were tested in the presence of Pluronic® P85, estradiol (E2; a inhibitor of BCRP) and verapamil (VP; a competitive inhibitor of P-gp). The tests were done at pH 5.5. Pluronic® P85 at a concentration of 0.2% showed a reduction in accumulation of TPT (0.6-fold) in cells expressing the BCRP transporter (Figure 3.8). However, Pluronic® P85 significantly enhanced 1.6-fold the accumulation of R123 in the same cells. E2 (10μM) cause a weak increase in the accumulation of TPT (1.33-fold), however it had almost no effect on R123 uptake. The presence of VP reduced the accumulation of R123 0.9-fold, and it enhanced accumulation of TPT 1.23-fold. Both E2 and VP were not statistically significant (respectively P=0.083 and P=0.3).

In another study, the accumulations of the anti-cancer drug mitoxantrone (MTX; a substrate of P-gp and BCRP) in MCF-7 sensitive, MCF-7 ADR and MCF-7 TPT cells were tested while exposing the cells to Pluronic® P85 (0.2%), VP (10μM) and E2 (100μM). The accumulation of MTX in cells was determined by the spectrophotometry (OD) at 660 nm. Pluronic® P85 significantly enhanced the accumulation of MTX in MCF-7 ADR cells but had no effect on transport of MTX in MCF-7 TPT cells (Figure 3.9). Pluronic® P85 increased the accumulation of MTX 2.7-fold, and VP increased it 2.3-fold. E2 had no

effect on accumulation of MTX in MCF-7 ADR and MCF-7 TPT cells. However, E2 demonstrated a 1.5-fold increase in MTX uptake by MCF-7 sensitive cells.

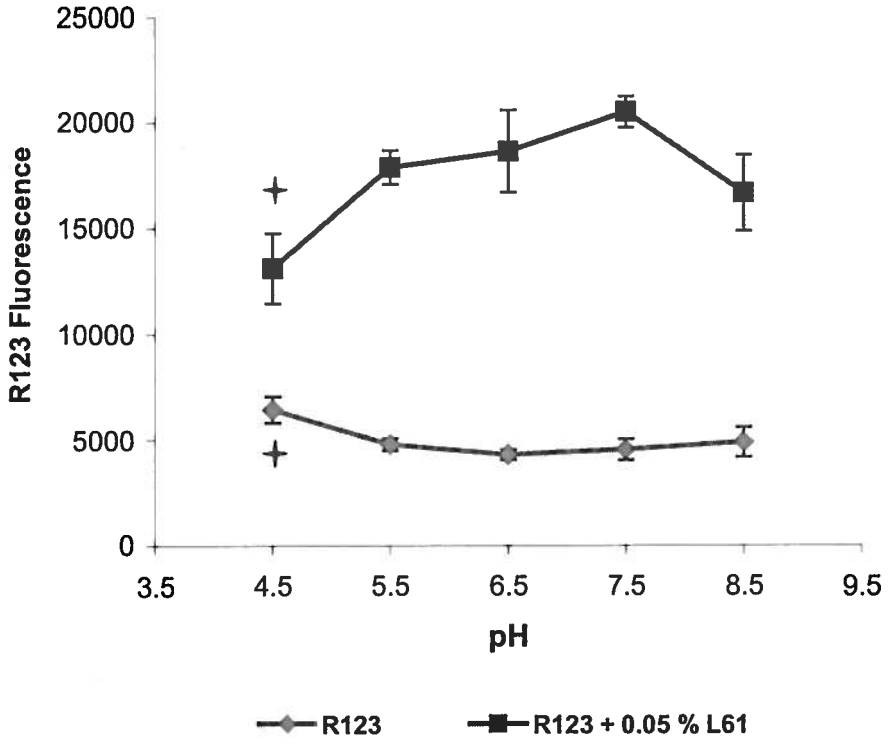


Figure 3.5 Effect of pH on the accumulation of R123 in MCF-7 ADR cells in the presence of Pluronic® L61.

The accumulation of R123 in MDR resistant cells MCF-7 ADR (expressing P-gp) was examined at pH 4.5, pH 5.5, pH 6.5. pH 7.5 and pH 8.5. (λ_{Ex}=485nm and λ_{Em}=530nm), both in the presence of 0.05% Pluronic® L61, and in the absence of polymer . The experiment was done in triplicates. A significant effect of pH on the accumulation of R123, as compared to the pH 7.5, was observed for both series only for pH 4.5.

+ Denotes results that are significantly different from those measured at pH 7.5 ($P \leq$ 0.05).

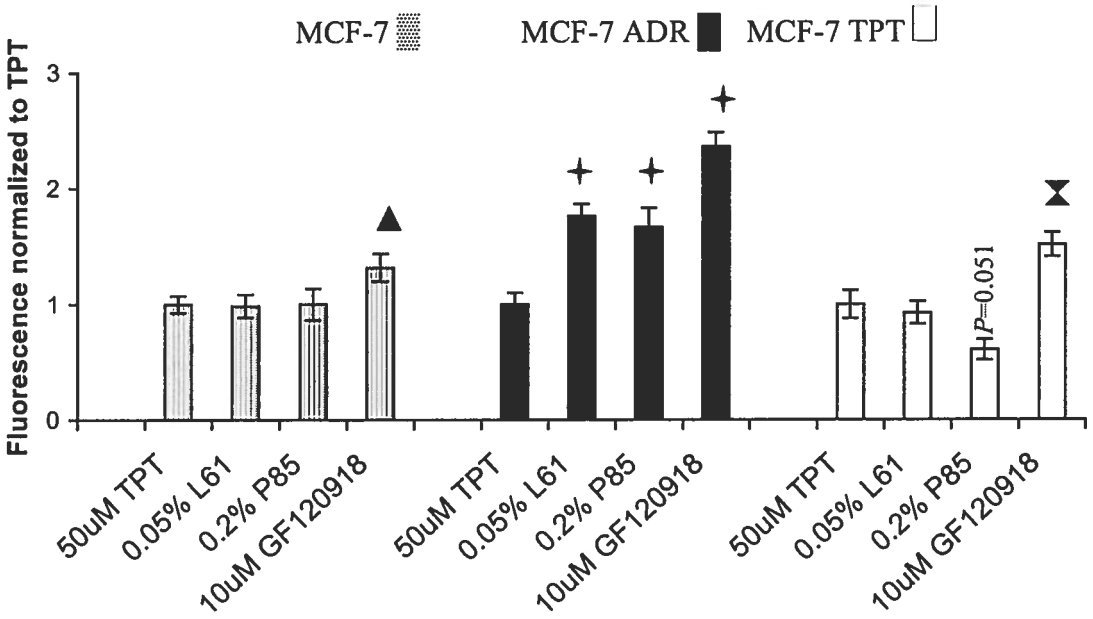


Figure 3.6 Accumulation of topotecan (TPT) in MCF-7, MCF-7 ADR and MCF-7 TPT cells in the presence of Pluronic® L61, Pluronic®P85, or GF120918.
The modulatory effect of Pluronic® L61 at 0.05% and Pluronic® P85 at 0.2% concentration on MDR resistant cells MCF-7 ADR (expressing P-gp), MCF-7 TPT (expressing BCRP) and their parental sensitive cells MCF-7 were examined for the accumulation of TPT. The experiment was performed at pH 5.5 to maintain TPT in the lactone form. The inhibitory effect of polymers was compared to the inhibitory effect of GF120918 (a non-specific inhibitor of P-gp and BCRP). TPT accumulation was measured by fluorescence plate reader (λ_{Ex}=360nm and λ_{Em}=530nm), and results were normalized for each cell line separately (fluorescence of samples with TPT alone equals 1). The experiment was done in triplicate. Pluronics® L61 and P85 were effective in increasing the accumulation of TPT only in MCF-7 ADR cells. GF120918 was found to be more potent than polymers in increasing the accumulation of TPT in P-gp and BCRP expressing cell lines.
▲,+, ⧗ Respectively denote for MCF-7, MCF-7 ADR and MCF-7 TPT cells results that are significantly different from TPT alone (control) ($P \leq 0.05$).

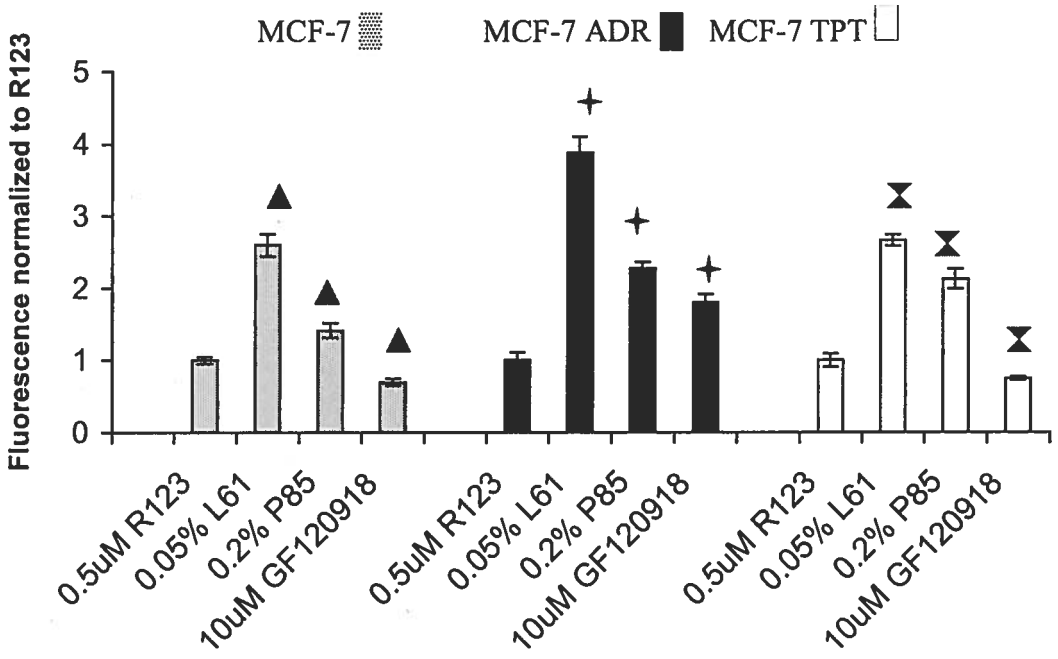


Figure 3.7 Accumulation of rhodamine (R123) in MCF-7, MCF-7 ADR and MCF-7 TPT cells in the presence of Pluronic® L61, Pluronic®P85, or GF120918. The modulatory effect of polymers L61 (0.05%) and P85 (0.2%) was tested on R123 accumulation in MDR resistant cells MCF-7 ADR (expressing P-gp), MCF-7 TPT (expressing BCRP), and their parental sensitive cells MCF-7. The experiment was performed at pH 5.5. The inhibitory effect of polymers was compared to that of GF120918 (a non-specific inhibitor of P-gp and BCRP). The accumulation of R123 was measured by fluorescence plate reader (λ_{Ex}=485nm and λ_{Em}=530nm), and all results were normalized for each cell line separately (fluorescence of samples with R123 alone equals 1). The experiment was repeated in triplicate. Pluronics® L61 and P85 enhanced the accumulation of R123 in both MCF-7 ADR and MCF-7 TPT cell lines. The sensitizing potency of polymers for the uptake of R123 on cells MCF-7 TPT was greater than that of GF120918.

▲,✦, ⧗ Respectively denote for MCF-7, MCF-7 ADR and MCF-7 TPT cells results that are significantly different from R123 alone (control) ($P \leq 0.05$).

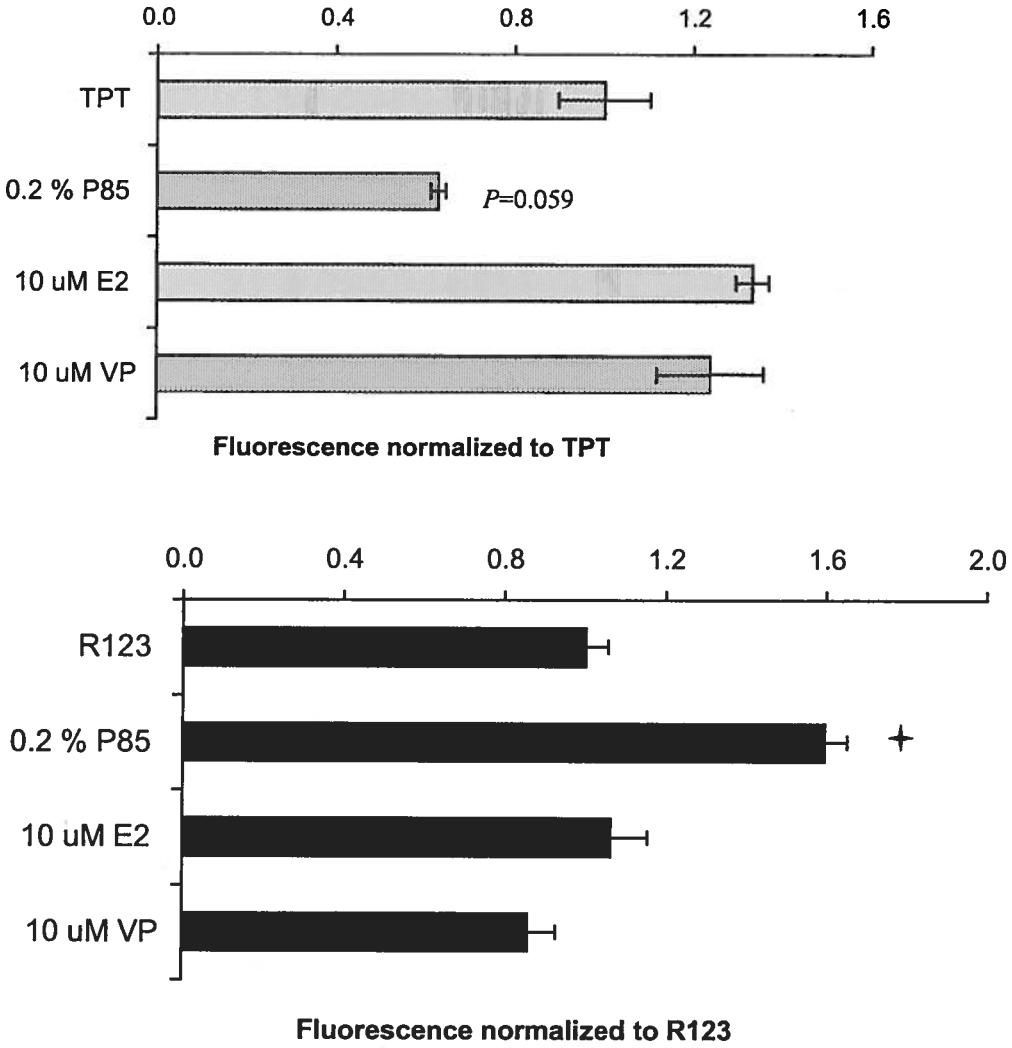


Figure 3.8 Accumulation of topotecan (TPT) and rhodamine (R123) in MCF-7 TPT cells in the presence of Pluronic® P85, estradiol (E2), or verapamil (VP).
The modulatory effect of Pluronic® P85 (0.2%) was tested on accumulation of TPT ▒ and R123 ■ MDR resistant cells. The experiment was performed at pH 5.5. The inhibitory effect of polymer was compared to the inhibitory effect of estradiol (E2; a BCRP inhibitor) and verapamil (VP; a competitive P-gp inhibitor). Accumulation was measured by fluorescence plate reader at (λ_{Ex}=360nm and λ_{Em}=530nm) for TPT and at (λ_{Ex}=485nm and λ_{Em}=530nm) for R123. Results were normalized (fluorescence of the samples with TPT alone or R123 alone equal 1, respectively). The experiment was repeated in triplicate. Pluronic® P85 enhanced the accumulation of R123 in cells expressing the BCRP transporter and it showed no effect on the accumulation of TPT on the same cells.
✦ Denotes results that are significantly different from R123 alone ($P \leq 0.05$).

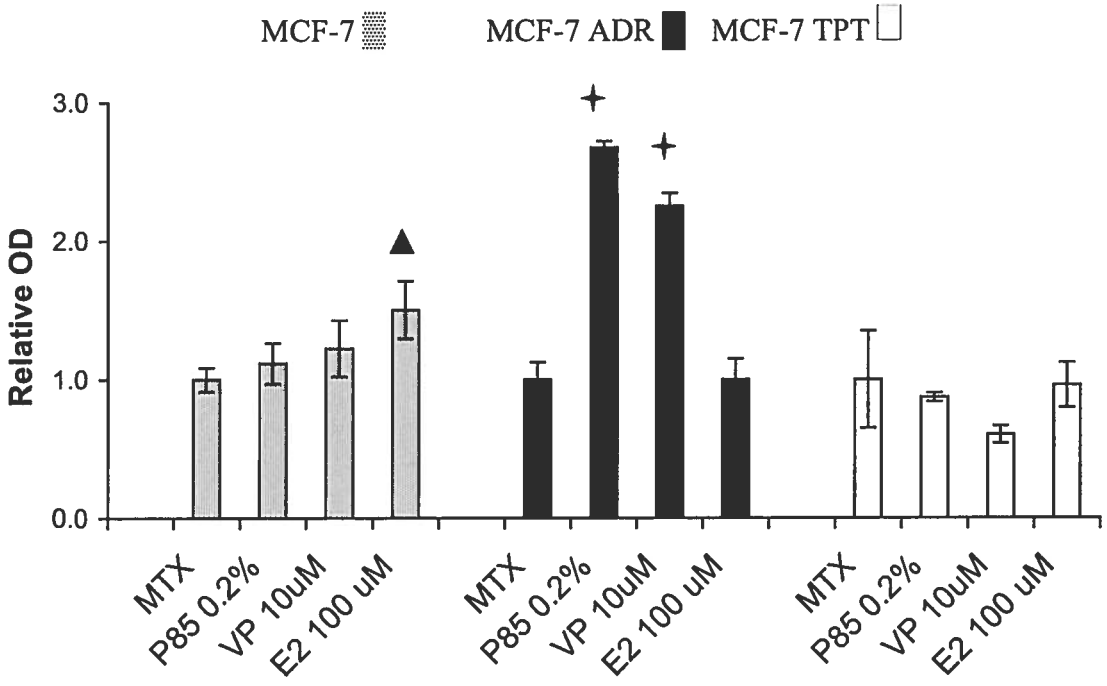


Figure 3.9 Accumulation of mitoxantrone (MTX) in MCF-7, MCF-7 ADR and MCF-7 TPT cells in the presence of Pluronic® P85, estradiol (E2), or verapamil (VP).

The modulatory effect of polymer P85 (0.2%) was tested on the accumulation of MTX in MCF-7 ADR (expressing P-gp) and MCF-7 TPT (expressing BCRP) MDR resistant and their parental sensitive cells MCF-7. The inhibitory effect of polymer Pluronic® P85 was compared to effect of E2 (inhibitor of BCRP) and VP (competitive inhibitor of P-gp). The accumulation of MTX was measured by its optical density (OD) at 660nm and results were normalized (MTX alone equals 1). The experiments were repeated in triplicate. Pluronic® P85 and VP were almost equally effective in increasing the accumulation of MTX in MCF-7 ADR cell lines.

▲,✦ Respectively denotes for MCF-7, MCF-7 ADR cells results that are significantly different from MTX alone (control) ($P \leq 0.05$).

3.2.4 *Effect of polymers on cells expressing the MRP1 transporter*

The effect of Pluronics® on MRP1 functional activity was evaluated. The accumulation of Dox, fluorescein, (both substrates of MRP1) and R123 (substrate of P-gp and BCRP) in HL60 ADR cells (expressing MRP1-mediated efflux system) and in the parental sensitive cells HL 60 was tested in the presence of Pluronic® L61 or Pluronic® P85.

The accumulation of Dox (20μM) in HL60 sensitive and in HL 60 ADR resistant cells in the presence Pluronic® L61 at various concentrations clearly showed that the polymer had no effect on the accumulation of Dox in the cells expressing MRP1 transporter (Figure 3.10). An elevated uptake value was observed for HL 60 ADR cells exposed to Pluronic® L61 at 0.2% concentration. However, such an observation is likely caused by polymer aggregation at this high concentration.

Pluronic® L61 did not enhance the accumulation of fluorescein (FL; 20μM) in HL 60 sensitive and HL 60 ADR resistant cells (Figure 3.11).

Accumulation of R123 (0.5μM) in HL 60 and in HL 60 ADR cells have showed a significant increase in accumulation of the probe in the presence of Pluronics® L61 and P85. The effect was concentration-dependent for both polymers but only Pluronic® L61 exhibited a maximum effect at moderate concentration, a bell-shaped curve on Fig 3.12. The enhancement of R123 uptake by polymers was detected for both sensitive and resistant cells (Figure 3.12). In HL 60 ADR cells, Pluronic® L61 was most effective at the concentration of 0.05% which is close to the CMC of this polymer. R123 accumulation in both cell lines formulated with Pluronic® P85 at various concentrations and in the presence of Pluronic® L61 at 0.05% were also tested. The observations suggest that Pluronic® P85 was equally active as a colloid in increasing the accumulation of R123. The uptake was concentration-dependent without a bell-shape curve.

The enhancing effect on R123 accumulation in both HL 60 sensitive and HL 60 ADR resistant cells by Pluronic® P85 was equal at concentrations of 0.5%, and 0.2% which suggests saturation. At 0.2% the accumulation of R123 in HL 60 sensitive and HL 60 ADR cells was respectively increased 4.2-fold and 3-fold, thus HL 60 sensitive cells were slightly more sensitized by Pluronic® P85 then resistant HL 60 ADR cells. Pluronic®

L61 (0.05 %) caused an almost 4.6-fold increase in accumulation of R123 for both cell lines. The above results may suggest that both sensitive and resistant cell lines express P-gp. To examine this assumption accumulation of R123 was evaluated in the presence of two P-gp inhibitors; VP and cyclosporin A.

The studies for the accumulation of R123 in HL 60 and in HL 560 ADR cells in the presence of Pluronic® L61 (0.05%), VP (24.5ug/ml), cyclosporin A (CSA; 20uM) and both VP and CSA mixed with Pluronic® L61 (0.05%) demonstrated that for both cell lines only Pluronic® L61 had an effect on the accumulation of R123. Neither of the cell lines reacted to the exposition to VP or CSA alone. Consequently, the results indicate that the observed Pluronic® effect in HL 60 and HL 60 ADR cells is not related to P-gp expression (Figure 3.13).

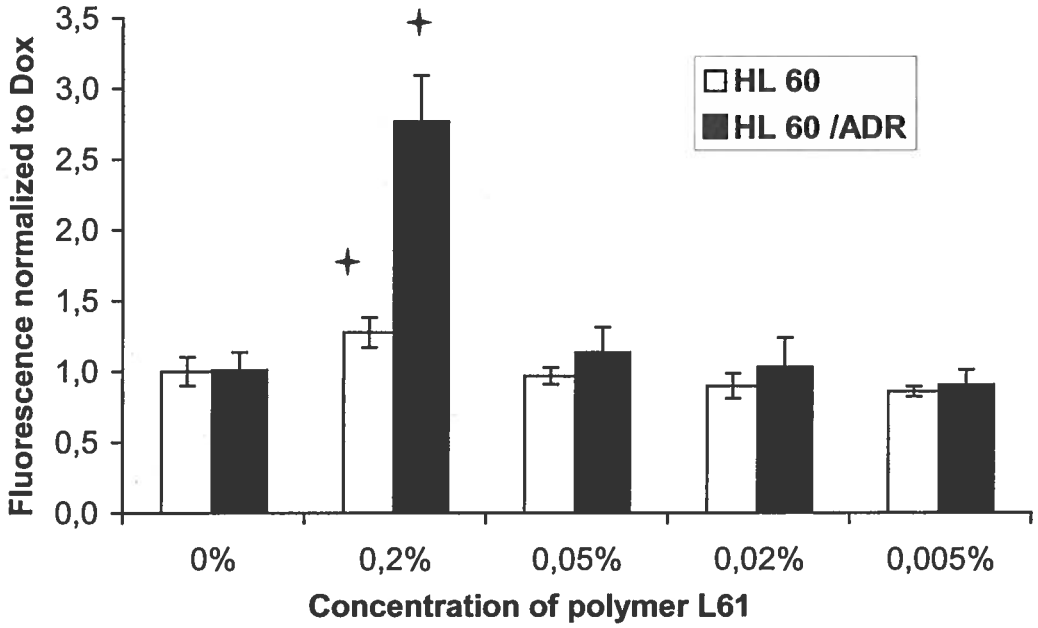


Figure 3.10 Accumulation of doxorubicin (Dox) in HL 60 sensitive and in HL 60 ADR cells in the presence of Pluronic® L61 at various concentrations.
The modulatory effect of Pluronic® L61 at various concentrations on MDR resistant HL 60 ADR (expressing MRP1 transporter) cells and their parental sensitive HL 60 cells were examined for the accumulation of Dox (20μM). Accumulation was measured by fluorescence plate reader at (λ_{Ex}=485nm and λ_{Em}=590nm). Results were normalized (fluorescence of samples with Dox alone equals 1). The experiment was repeated in triplicate. Pluronic® L61 showed no effect on the accumulation of Dox in HL 60 ADR cells. + Denotes results that are significantly different from Dox alone ($P \leq 0.05$).

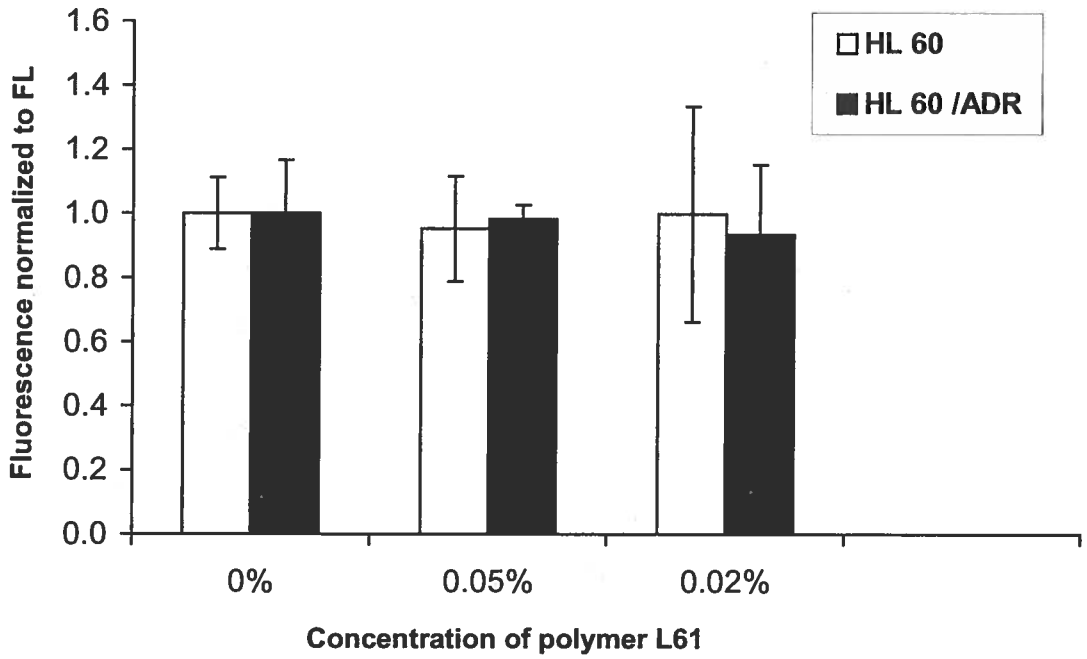


Figure 3.11 Accumulation of Fluorescein (FL) in HL 60 sensitive and in HL 60 ADR cells in the presence of Pluronic® L61 at various concentrations.
The modulatory effect of Pluronic® L61 at various concentrations on MDR resistant HL 60 ADR (expressing MRP1 transporter) cells and their parental sensitive HL 60 cells were examined for the accumulation of FL (20μM). Accumulation was measured by fluorescence plate reader at (λ_{Ex}=485nm and λ_{Em}=530nm). Results were normalized (fluorescence of samples with FL alone equals 1) and the experiment was repeated in triplicate. Pluronic® L61 showed no significant (P>0.05) effect on the accumulation of FL in HL 60 ADR cells.

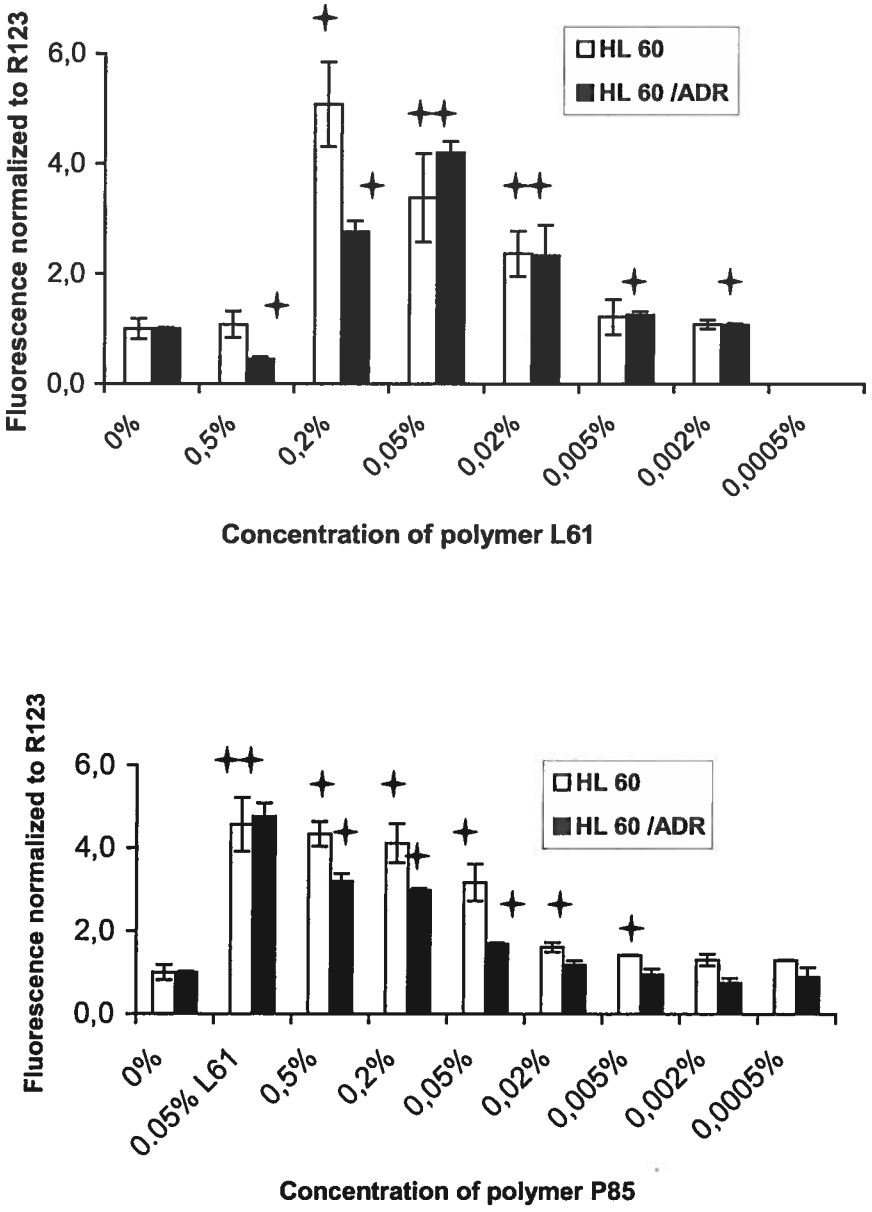


Figure 3.12 Accumulation of rhodamine 123 (R123) in HL 60 sensitive and in HL 60 ADR cells in the presence of Pluronic® L61 or Pluronic® P85 at various concentrations.

The modulatory effect of Pluronic® L61 and P85 at various concentrations on R123 accumulation in MDR resistant HL 60 ADR (expressing MRP1 transporter) cells and their parental sensitive HL 60 cells were examined by measurements of R123 fluorescence by plate reader at (λ_{Ex}=485nm and λ_{Em}=530nm). All results were normalized (fluorescence of samples with R123 alone equals 1). The experiment was repeated in triplicate. The accumulation of R123 in the presence of Pluronic® L61 or P85 was concentration-dependent. Polymers sensitized both the sensitive and the drug resistant cells. + Denotes results that are significantly ($P \leq 0.05$) different from R123 alone.

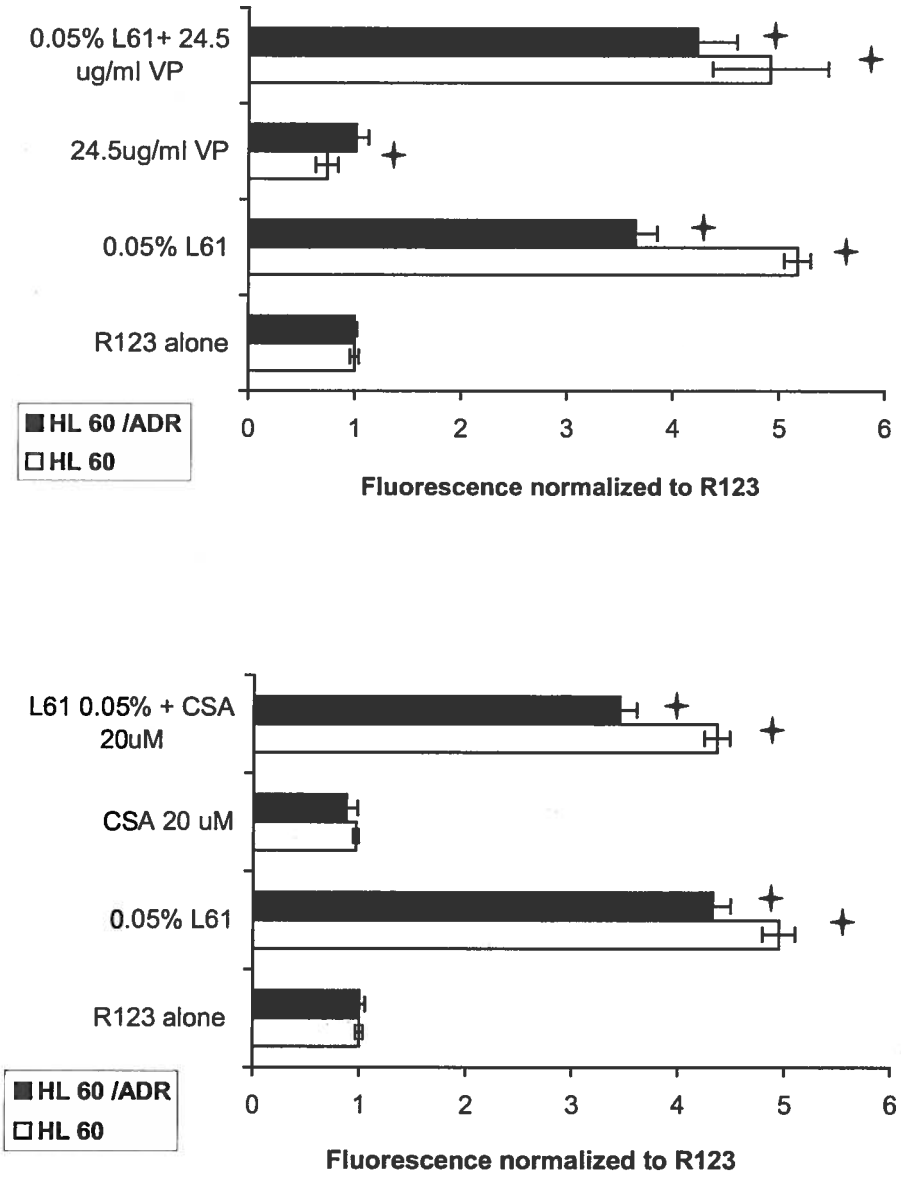


Figure 3.13 Accumulation of rhodamine 123 (R123) in HL 60 sensitive and in HL 60 ADR cells in the presence of Pluronic® L61, cyclosporin A (CSA), or verapamil (VP).

The modulatory effect of Pluronic® L61 (0.05%), CSA (20μM) and VP (24.5 μg/ml) on MDR resistant HL 60 ADR (expressing MRP1 transporter) cells and their parental sensitive HL 60 cells were examined for the accumulation of R123. The accumulation was measured by fluorescence plate reader at (λ_{Ex}=485nm and λ_{Em}=530nm). Results were normalized (fluorescence of samples with R123 alone equals 1) and the experiment was repeated in triplicate. Pluronic® L61 sensitized both sensitive and resistant cell lines. VP and CSA alone showed no effect on the accumulation of R123. + Denotes results that are significantly different from R123 alone ($P \leq 0.05$).

3.3 Effects of Pluronics® on cytotoxicity of doxorubicin (DOX) and topotecan (TPT)

The effects of Pluronics® on cytotoxicity of the anticancer drugs doxorubicin (DOX) and topotecan (TPT) were tested on drug resistant cells. Doxorubicin-resistant, MCF-7 ADR (expressing P-gp transporter), HL 60 ADR (expressing MRP1 transporter) and MCF-7 TPT (expressing BCRP transporter) cells, and their respective parental sensitive MCF-7 cells and HL 60 cells were incubated with increasing concentrations of doxorubicin (Dox) or topotecan (TPT), alone or in the presence of 0.05% Pluronic®L61, for two hours. The experiments with Dox were done at the standard pH of the culture medium (approximately at pH 7.5). However in the case of TPT, the cytotoxic drug treatment was done at pH 5.5 to maintain the lactone form of TPT. Cells were re-incubated with medium at regular pH for three days. Finally, the XTT colorimetric assay was utilized to measure the viability of the cells. The IC_{50} corresponding to the concentration of Dox or TPT inhibiting 50% of cell proliferation was determined (Table 3.2). The results show that Pluronic® L61 at 0.05% reduced the IC_{50} of doxorubicin in both MCF-7 ADR resistant and MCF-7 sensitive cells (Figure 3.14). In doxorubicin resistant MCF-7 ADR cells, the IC_{50} of Dox was reduced from 147 000 ng/ml to 800 ng/ml. In MCF-7 sensitive cells the IC_{50} of Dox was reduced from 1200 ng/ml to 500 ng/ml. Thus, Pluronic® L61 sensitized the MCF-7 ADR cells (resistant to doxorubicin) 184-fold, compared to a 2.4-fold sensitization of the parental MCF-7 drug-sensitive cells. Hence, based on these results, resistance to Dox was reduced from a 123 to 1.6 when exposed to polymer.

HL 60 ADR (doxorubicin resistant cells, expressing MRP1 transporter) were also sensitized by Pluronic® L61 (Figure 3.15). The IC_{50} of Dox for HL 60 ADR cells, was reduced from 15 000 ng/ml to 500 ng/ml in the presence of Pluronic® L61 at 0.05% concentration (Table 3.2). No sensitizing activity was observed on HL 60 sensitive cells when these cells were exposed to the same polymer. The IC_{50} of Dox was found to increase from 30 ng/ml to 50 ng/ml in the presence of Pluronic® L61, which is not a significant change. Thus, Pluronic® L61 was found to increase the sensitivity of HL 60 ADR resistant cells to doxorubicin by 30 times. The resistance factor for Dox was reduced from 500 times to 10 times when cells were exposed to Pluronic® L61 (0.05%).

Pluronic® L61 showed no sensitizing effect on MCF-7 TPT (topotecan resistant cells, expressing BCRP), and MCF-7 sensitive cells, to increase the cytotoxicity of TPT (Figure 3.14). The IC_{50} of TPT on MCF-7 TPT resistant cells was 53 000 ng/ml, and 76 000 ng/ml in the presence of 0.05% Pluronic® L61.The IC_{50} of TPT on MCF-7 sensitive cells IC_{50} of TPT was reduced from 2900 ng/ml to 2600 ng/ml in the presence of 0.05% Pluronic® L61 (Table 3.2). The observed changes of IC_{50} caused by the polymer were negligible.

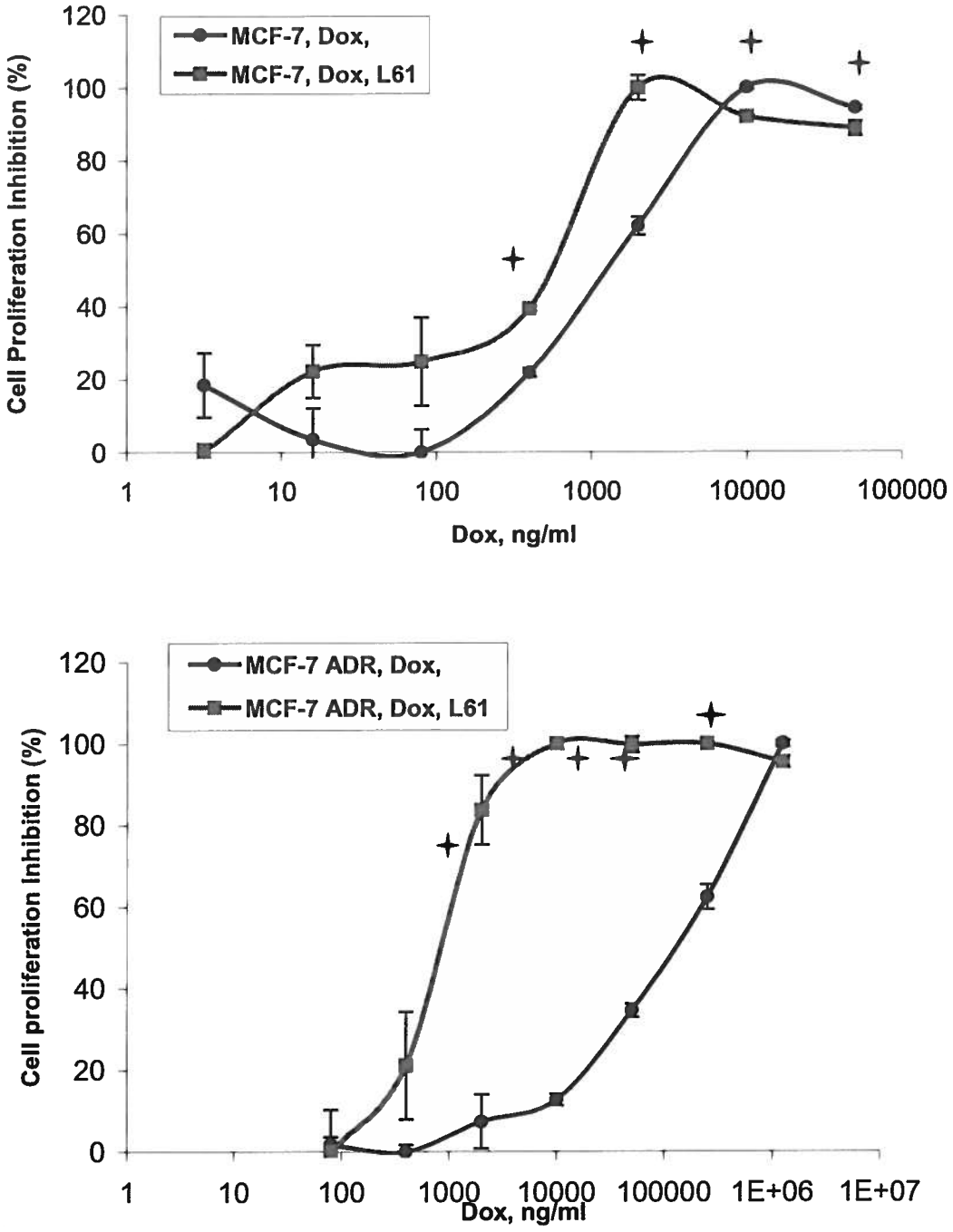


Figure 3.14 Cytotoxic effect of doxorubicin (Dox) on MCF-7 sensitive and MCF-7 ADR resistant cell lines in the presence or absence of Pluronic® L61.

The graph presents cell proliferation inhibition (%) versus increasing Dox concentration (ng/ml), alone or with 0.05% Pluronic® L61. Cells were incubated with Dox for 2 hours. The experiment was done in triplicate. The cytotoxic effects of Dox were determined using the XTT assay. Pluronic® L61 caused a greater sensitization effect on MCF-7 ADR resistant cells (expressing P-gp transporter) compared to MCF-7 sensitive cells which showed a slight effect for the enhancement of Dox cytotoxicity. + Denotes concentrations at which the results obtained with or without Pluronic® L61 are significantly different ($P \leq 0.05$).

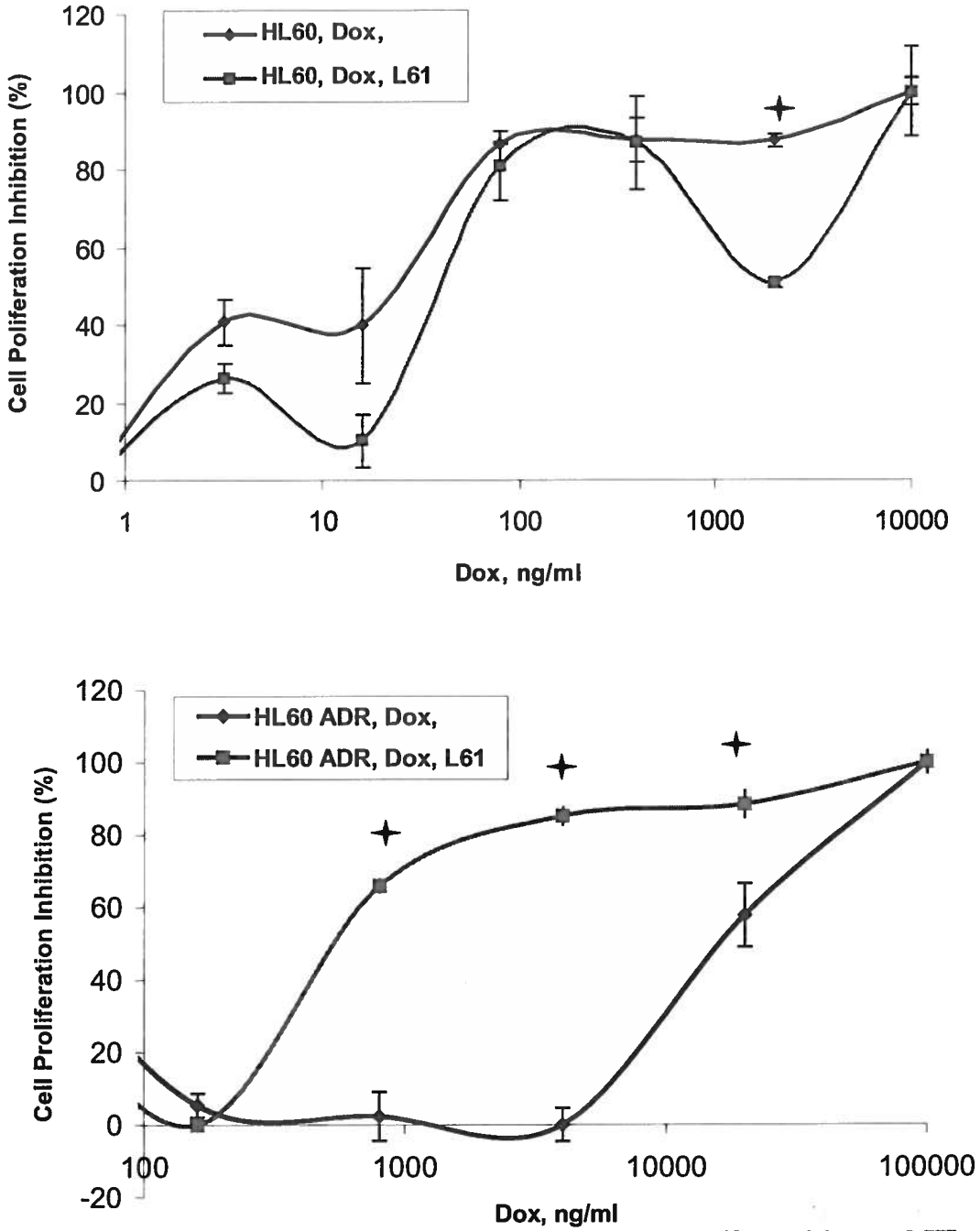
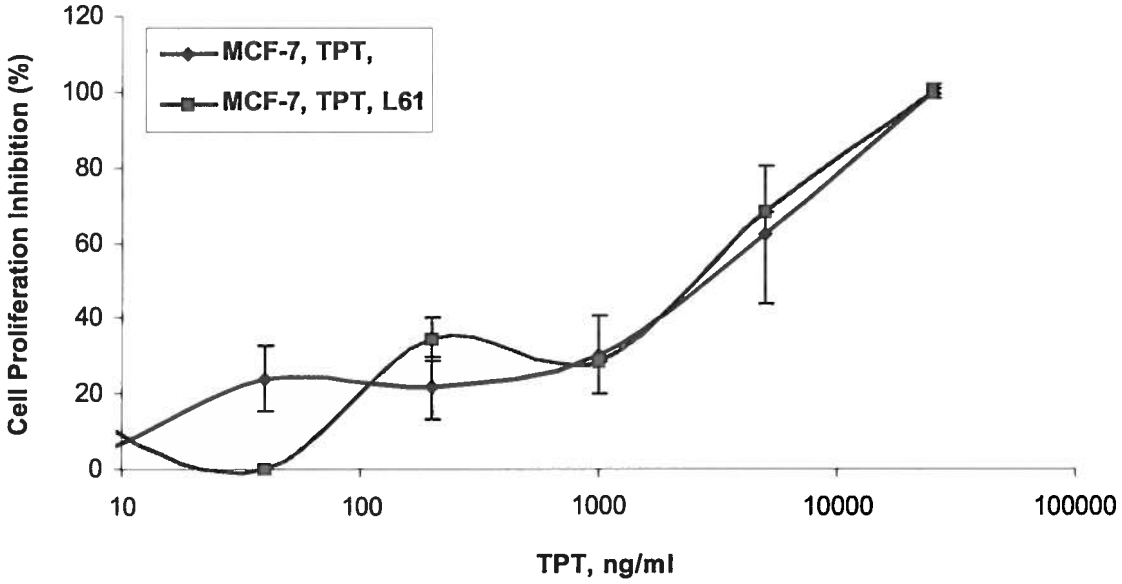


Figure 3.15 Cytotoxic effect of doxorubicin (Dox) on HL 60 sensitive and HL 60 ADR resistant cell lines in the presence or absence of Pluronic® L61.

The graph presents cell proliferation inhibition (%) versus increasing Dox concentration (ng/ml) alone, and when formulated with 0.05% Pluronic® L61. Cells were incubated with Dox for 2 hours. The experiment was done in triplicate. The cytotoxic effects of Dox were determined using the XTT assay. Pluronic® L61 sensitized HL 60 ADR resistant cells (expressing MRP1 transporter) increased the cytotoxic effect of Dox. No sensitization to doxorubicin was observed when HL 60 sensitive cells exposed to Pluronic® L61.

✦ Denotes concentrations at which the results obtained with or without Pluronic® L61 are significantly different ($P \leq 0.05$).



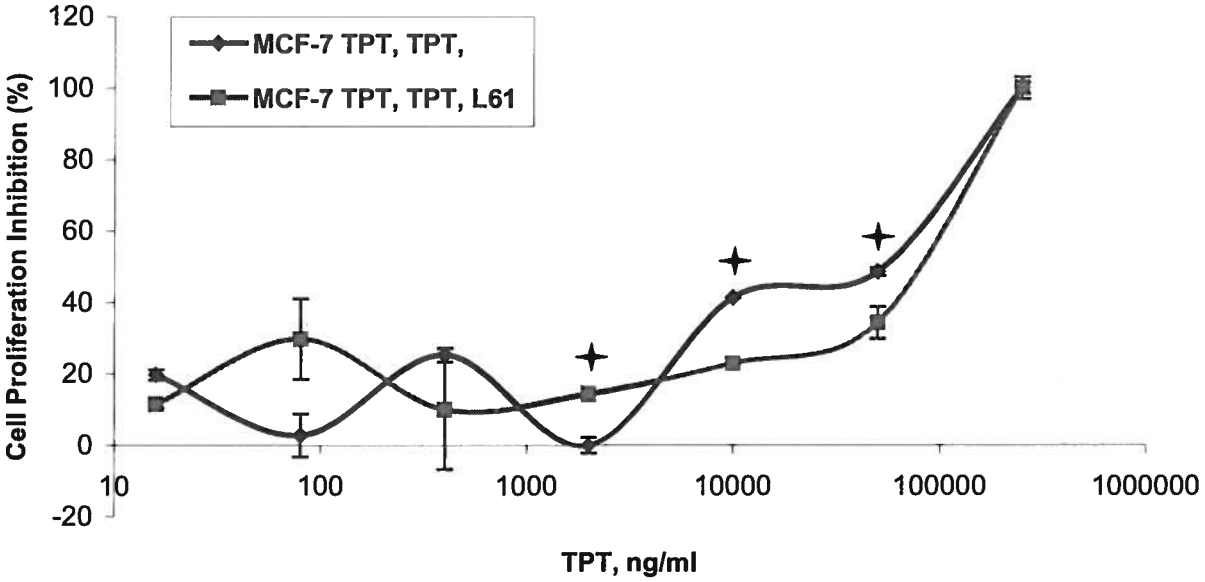


Figure 3.16 Cytotoxic effect of topotecan (TPT) on MCF-7 sensitive and MCF-7 TPT resistant cell lines in the presence or absence of Pluronic® L61.

The graph presents cell proliferation inhibition (%) versus increasing TPT concentration (ng/ml) alone or with 0.05% Pluronic® L61. Cells were incubated with TPT for 2 hours at pH 5.5, then re-incubated in medium at regular pH for three days. The experiment was done in triplicates. The cytotoxic effects of TPT were determined using the XTT assay. Pluronic® L61 had no TPT sensitizing effect on MCF-7 TPT cells (expressing BCRP transporter).

✦ Denotes concentrations at which the results obtained with or without Pluronic® L61 are significantly different ($P \leq 0.05$).

Table 3.2 Cytotoxic activity of doxorubicin (Dox) or topotecan (TPT) alone and in the presence of Pluronic® L61 at 0.05% concentration on three different MDR cell lines and their respective parental sensitive cells

Cell lines	Drug IC_{50} (ng/ml)*	Drug + 0.05% L61 IC_{50} (ng/ml)*	Sensitizing activity of L61 at 0.05%† (number of times)	Resistance factor of drug‡	Resistance factor of drug + 0.05% L61‡
MCF-7 / Dox	1200 ± 50	500 ± 20	2.4		
MCF-7 ADR / Dox	147 000 ± 8900	800 ± 50	184	123	1.6
HL 60 / Dox	30 ± 4	50 ± 6	0.6		
HL60 ADR / Dox	15 000 ± 5000	500 ± 80	30	500	10
MCF-7 / TPT	2900 ± 1400	2600 ± 80	1.1		
MCF-7TPT / TPT	53 000 ± 2300	76 000 ± 3300	0.7	18	29

* IC_{50} indicates the concentration of doxorubicin (Dox) or topotecan (TPT) at which the proliferation of cells is inhibited by 50%. The table lists the mean result of three experiments (ng/ml) ± SEM values.
† The ratio between IC_{50} of drug (Dox or TPT) and IC_{50} of drug (Dox or TPT) + 0.05% Pluronic® L61 formulation.
‡Calculated by dividing the value obtained from IC_{50} for resistant cells by the IC_{50} for sensitive parental cell lines for drug (Dox or TPT) and for drug (Dox or TPT) + 0.05% Pluronic® L61.

CHAPTER 4: Discussion

Previous studies have demonstrated that certain Pluronic® block copolymers sensitize various MDR resistant cancer cells overexpressing P-gp and MRP drug efflux systems (Alakhov et *al*, 1996; Venne et *al.*, 1996; Miller et *al.*, 1999). The objective of the present study was to examine the inhibitory effects of several Pluronics® on P-gp, MRP1-overexpressing cells and for the first time to evaluate whether Pluronics® could be effective to overcome BCRP-mediated drug resistance. Additionally, the study also included a comparison of the inhibitory potency of Pluronics® to that of other known MDR modulators.

4.1 Effect of Pluronics® on cells overexpressing the P-gp transporter

As previously demonstrated, certain Pluronics® effectively inhibit P-gp mediated drug efflux and increase accumulation of P-gp substrates in cells (Venne et *al.*, 1996; Batrakova et *al.*, 1998). This is a combined effect of elevated influx and decreased efflux (Venne et *al.*, 1996). We have found that Pluronics® L61, L92 and P123 at their respective maximum effective concentration increased the accumulation of R123 in MCF-7 ADR cells (expressing P-gp) more than the less hydrophobic Pluronic® P85. The differences among Pluronics® L92, P123 and Pluronics® L61 were not statistically significant. The magnitude of the accumulation enhancement effect was dependent on the concentration of polymers, and showed maximums at 0.2% for L61, and 0.5% for P85, approximately 3 and 10 times above the measured CMC, respectively. We believe that these results are not contradicting previously published hypotheses, which state that the unimeric (non-aggregated) polymers are responsible for efflux inhibition. The aggregation of amphiphilic polymers and formation of micelles is a complex process. The transition from unimers to micelles occurs at a broad range of concentrations, and certain amounts of free (non-incorporated in micelles) polymer molecules are present in polymer solutions at concentrations above CMC, in equilibrium with micelles (Kabanov et *al.*, 1995). It is also possible that the cells interact with extracellular polymeric micelles, and may locally change the concentration of unimers available for membrane fluidization. On the other hand, due to limitations of polymer diffusion, and due to the presence of various

amphiphilic structures (membranes), and other macromolecules (proteins) inside cells, micelle formation inside cells is unlikely.

The increase of accumulation in MCF-7 ADR cells in the presence of Pluronics® L61 and P85 has also been observed for Dox. Similarly to R123 accumulation, Pluronic® L61 proved to be more potent than Pluronic® P85 in enhancing the accumulation of Dox. However, the dependence of the effect on concentration of polymers for Dox accumulation was somewhat different than in the case of R123 accumulation. The highest tested concentrations of each Pluronic®, up to 2%, proved to be still effective. This may suggest that a different mechanism of accumulation and efflux may be possible for Dox and for R123. Regarding that, the possibility of a contribution of passive diffusion of the probes has been ruled out by testing probe accumulation at 4°C (a temperature at which P-gp-efflux is inactive). No effect of polymers was noted under these conditions (data not shown). One possible mechanism differing Dox and R123 uptake may be related to Dox absorption in cells. According to literature the resistance of MCF-7 ADR cells to Dox is associated with sequestration of the drug within cytoplasmic vesicles caused by the protonation of drug molecules and a decrease in pH inside the vesicle (Altan et *al.*, 1998). A previous study has demonstrated that Pluronics® act as an ionophore, and they facilitate the liberation of Dox sequestered within vesicles (Venne et *al.*, 1996). Pluronic® L61 enhanced the cytotoxicity of Dox and proved to be highly effective in sensitizing P-gp overexpressing cells (184-fold increase of IC_{50} of Dox for MCF-7 ADR) compared to Pgp sensitive cells (2.4 -fold increase of IC_{50} of Dox for MCF-7). The results were consistent with previous studies (Venne et *al.*, 1996; Batrakova et *al.*, 1999), however in our studies hypersensitization (resistance factor lower than 1) was not achieved. We estimate that MCF-7 ADR cells resistance to Dox when exposed to 0.05% Pluronic® L61 to be approximately 1.6- fold more sensitive. We also noted an increase of accumulation of two other cytotoxic drugs, TPT and MTX in MCF-7 ADR cells exposed to either Pluronic® L61 or Pluronic® P85.

Overall, the accumulation and cytotoxicity studies demonstrate that the tested Pluronics® are very effective in inhibiting P-gp-mediated drug efflux.

4.2 Effect of Pluronics® on cells overexpressing the BCRP transporter

Pluronics® showed only limited effectivness in inhibiting BCRP-mediated efflux. The accumulation of TPT in MCF-7 TPT cells was not increased by Pluronics® L61 or P85; the latter even decreased TPT accumulation. Both polymers increased R123 accumulation in the same cells; however, a similar increase in R123 accumulation was occasionally observed in MCF-7 parental cells, and therefore it is not clear if the observed effect can be related to BCRP overexpression in MCF-7 TPT cells. For the comparison, GF120918, a P-gp inhibitor did not produce any effect on R123 accumulation in MCF-7 TPT and in parental MCF-7 cells, suggesting that the observed effect of polymers is not related to co-expression of P-gp in these cell lines.

Pluronic® L61 showed no sensitizing effect on TPT resistant MCF-7 TPT cells; the cytotoxicity of TPT remained virtually unchanged. This result is in good accord with above accumulation studies for TPT.

4.3 Effect of Pluronics® on cells overexpressing the MRP1 transporter

Exposure of MRP1 overexpressing cells (HL 60 ADR) to Pluronic® L61 did not affect the accumulation of FL, a well-known MRP1 substrate. Accumulation of another substrate, Dox was affected by a 0.2% solution of Pluronic® L61, but not by lower concentrations. Both Pluronics® L61 and P85 significantly increased the accumulation of R123 in HL 60 ADR, and also in the doxorubicin sensitive parental HL 60 cells. This effect was concentration-dependent. It is known that R123 is a substrate of both P-gp and MRP1 (Zaman et *al.*, 1994; Minderman *et al.*, 1996).

The described observation raised the possibility that both HL 60 sensitive and HL 60 ADR cells used to test the inhibitory effect of Pluronic® on the MRP1 efflux system, might also overexpress P-gp. Therefore R123 accumulation was also studied in the presence of VP and CSA, two competitive inhibitors of P-gp, with or without the presence of Pluronic®. The lack of any effect of VP or CSA indicated that P-gp might not be responsible for the enhancement of R123 accumulation in the presence of polymers.

The possibility of contribution of passive diffusion of R123 was ruled out by testing accumulation at 4°C. Under these conditions, no active transport occurs; we observed that at 4°C the polymers did not affect R123 accumulation in HL 60 or HL60 ADR cells (data not shown).

Pluronic® L61 enhanced the cytotoxicity of Dox for HL 60 ADR cells, overexpressing MRP-1. We noted a 30-fold increase of IC_{50} of Dox for HL 60 ADR. This effect may be related to the only limited response of Dox accumulation in HL-60 ADR on the presence of Pluronic® L61 (0.2%), but further detailed studies may be required to confirm the correlation. We should also consider other causes of sensitization, such as involvement of other transporters, or involvement of another resistance mechanism.
The sensitization effect obseved certainly was less marked than the effect of Pluronic® L61 on P-gp overexpressing MCF-7 ADR cells.

4.4 Correlation between the structure and the bioactivity of Pluronics®

The previous studies have demonstrated that Pluronics® with highest efficacy in sensitizing P-gp overexpressing cells are those composed of an intermediate length of POP hydrophobic residues (30-60 units) and a HLB of less than 20, such as Pluronic® L61 or P85 (Batrakova et *al.*, 1999, Batrakova et *al,* 2003). This effect is likely related to the mode of interaction of Pluronic® molecules with lipid bilayers. The central hydrophobic part of Pluronic® molecules is incorporated into lipid bilayers, and peripheral hydrophilic chains stay outside of the bilayers; therefore only molecules having an appropriate hydrophobic-hydrophilic balance can be transported into the cells (Batrakova et *al.*, 2003).

Our studies on R123 accumulation in P-gp overexpressing cells are consistent with the literature. Pluronics® L61, L92, P85 proved to be powerful enhancing agents whereas Pluronics® F127, F38, L44 were inactive. In our studies on P-gp overexpressing MCF-7 ADR cells, Pluronic® L92 (MW=3650; number of blocks of propylene oxide: 50; HLB: =6), which has a longer POP, was more efficacious than Pluronic® L61 (MW=2000; number of block of propylene oxide: 31; HLB=3). Pluronic® L92 was also more effective than a more hydrophile-lipophile balanced, less hydrophobic Pluronic® P85 (MW=4600; number of block of propylene oxide: 40; HLB=16). Also, in the BCRP overexpressing cells MCF-7 TPT, Pluronic® L61 caused greater accumulation of R123 than Pluronic® P85.

The studies on R123 accumulation in HL 60 ADR cells (expressing MRP1), and in HL 60 sensitive cells (non-MRP1 overexpressing) did not show a clear distinction between the effect of Pluronic® L61 and Pluronic® P85. However, we observed a different type of dependence of the effect on polymer concentration. The bell-shaped curve was only observed for HL 60 ADR cells in the presence of Pluronic® L61. The lack of such a curve in other experiments, including the experiments done in the presence of Pluronic® P85 may suggest that both HL 60 ADR cells and their parent HL 60 cells are prone to interact with polymeric micelles, as well as with unimers. However, low sensitivity of HL 60 ADR to the highest tested concentration of Pluronic® L61 suggest that perhaps very hydrophobic Pluronics® such as L61 may be effective against the MRP1 efflux system. This possibility needs further investigation. For example the effect of different Pluronics® with various

structural compositions could be investigated to determine whether there is a structural-functional relationship for the inhibition of MRP1-efflux systems.

In other studies in MRP1 and MRP2- transfected cells Madin Darby Canine Kidney (MDCKII), and in Dox resistant MRP1 overexpressing COR-L23\R lung cancer cells, it was demonstrated that Pluronic® P85 enhanced the accumulation of vinblastine and doxorubicin. However Pluronic® P85 had no effect in increasing Dox cytotoxicity in COR-L23/R resistant cells (Batrakova et *al.*, 2003).

4.5 Substrate selectivity of P-gp, BCRP and MRP1 efflux systems exposed to Pluronics®

The mechanism by which Pluronics® are capable of sensitizing MDR cells overexpressing P-gp and MRP1 has not yet been fully explained. Previous studies have demonstrated that Pluronics® affect MDR cells overexpressing these transporters by increasing membrane fluidization, targeting mitochondria, and interfering with ATP-synthesis which results in ATP depletion. ATP is essential for the proper function of efflux proteins by ABC efflux systems (Slepnev et *al.*, 1992; Batrakova et *al.*, 2001). In addition, Pluronics® decrease intracellular levels of reduced GSH which is necessary for the GSH/GST detoxification system (Batrakova et *al.*, 2003). The key observations made in this work are related to the substrate selectivity of P-gp, MRP1 and BCRP efflux systems inhibited by Pluronics®, and other inhibitors. A summary concerning these observations is presented in Table 4.1.

Pluronics® show high inhibitory effects on P-gp efflux systems and are effective in increasing the accumulation of all tested substrates: R123, Dox, MTX and TPT. Pluronics® increased accumulation of R123 in BCRP expressing cells (MCF-7 TPT), but did not enhance accumulation of TPT. On the contrary, Pluronic® P85, but not Pluronic® L61, caused some decrease (60% of the initial value) of TPT accumulation; this result was not statistically significant (P=0.06), and its causes are not known. E2, a known BCRP-inhibitor caused a weak enhancement (though not statistically significant, P=0.083) of TPT accumulation in BCRP-overexpressing cells. The result correlates with the findings by (Imai et *al.* 2005), who demonstrated that E2 increases the accumulation of TPT and also found reduces BCRP expression in BCRP transduced MCF-7 /BCRP cells .

The only tested substance, which, significantly increased TPT accumulation in MCF-7 TPT is GF120918. The effect of GF120918, a non-specific P-gp and BCRP inhibitor was found to be transporter and substrate specific. GF120918 was more potent than Pluronics® L61 or P85 in enhancing the uptake of TPT in both MCF-7 and MCF-7 ADR cells. However, GF120918 was less effective than Pluronics® in increasing the accumulation of R123 in both cell lines. MCF-7 TPT cells are known to slightly overexpress P-gp (private communication by Supratek Pharma), and therefore it was necessary to evaluate if the described results might be caused by P-gp. This possibility has

been ruled out by the experiment with VP, another potent P-gp inhibitor included in this study. VP caused no effect on accumulation of R123 or TPT in MCF-7 TPT cells.

Pluronics® enhanced accumulation of R123, and slightly increased accumulation of Dox, but not FL in HL60 ADR cells overexpressing MRP1. Moreover, a similar increased accumulation of R123 was observed in ADR sensitive HL 60 cells, which do not overexpress MRP1. With the exception of Pluronic® L61, the concentration dependence of this effect did not show a maximum close to CMC. The cause of these findings is unclear and requires further research.

In the future the use of specific expression or inhibition (expression vector, siRNA) techniques would be essential in order to correlate the effects of Pluronics® with transporter specificity. Those studies could then help to characterize the contribution of transporters on substrate accumulation and selectivity in the presence of Pluronics®.

Table 4.1 The accumulation of substrates of efflux systems in P-gp, BCRP and MRP1 overexpressing cells (MCF-7 ADR, MCF-7 TPT and HL 60 ADR respectively) is selectively enhanced by the tested inhibitors

	P-gp[a]	BCRP[a]	MRP1[a]
Pluronic® L61[b]	R123, Dox, MTX, TPT	R123	R123
Pluronic® P85[b]	R123, Dox, MTX, TPT	R123	R123
GF120918[b]	R123, TPT	TPT	
VP[b]	R123		
E2[b]			

The table presents the summary of results obtained for accumulation experiments. The accumulation of the listed probes in the cells using P-gp, BCRP or MRP1 mediated efflux (MCF-7 ADR, MCF-7 TPT and HL 60 ADR, respectively) was enhanced selectively in the presence of the respective inhibitors.
Tested substrates included: R123, Dox, FL, MTX and TPT. FL was only tested with HL 60 and HL60 ADR cells in the presence of Pluronics® L61 or P85, and polymers showed no effect on probe accumulation.
Note: In our studies E2, caused a weak enhancement (not statistically significant, P=0.083) of TPT accumulation in BCRP-overexpressing cells.
[a] ABC-transporters
[b] Inhibitors

4.6 Relationship between P-gp, BCRP and MRP1 membrane structure and the inhibitory potency of Pluronics®

Our studies have demonstrated that the inhibitory effect of moderately hydrophobic Pluronics® on efflux systems is most pronounced on P-gp, and that there is a limited effect on MRP1 and BCRP-mediated efflux. The reason for such differences is unknown; however results published by various scientific groups give some insight into the efflux processes and effects of inhibitors, and may suggest directions for the design of future studies. A previous study on P-gp and MRP1 efflux systems exposed to Pluronic® P85 suggest that the different response of efflux systems to Pluronics® may be caused by higher ATPase activity in P-gp and lower ATPase activity in MRP1 (Batrakova et *al.*, 2004). It was suggested that Pluronics® which are able to incorporate into the lipid bilayer increase the membrane fluidity surrounding P-gp and MRP1 proteins without binding directly to the protein (Batrakova et *al.*, 2003). The changes within the membrane may cause conformational changes in NBDs that hinder ATP binding, and/or steric obstruction of the substrate binding sites of the protein (Batrakova et *al.*, 2004). The affinity of the NBF towards ATP is reduced when the intracellular membrane fluidity increases upon heating, which suggests a strong relationship between lipid membrane structure and the transporters NBD's (Sharom et *al.*, 1999; Lu and Liu, 2001). The different effect of Pluronics® on the three studied efflux systems may also be related to structural discrepancies of the transporter proteins. P-gp is a protein made of two analogous halves containing 2 NBF's for ATP binding (Chen *et al.*, 1986). Recent data by Takada et *al.*, suggest that a proper interaction between NBF's is essential for the function of P-gp; even though both NBF's hydrolyze ATP alternatively, they cooperate in functional transport activity. Hindering ATP binding at the NBF that responsible for the transport of substrates may have a direct effect on the other NBF that is responsible to restore the protein to a high affinity substrate binding state for the next transport cycle (Dey et *al.*, 1997; Takada et *al.*, 2000).

The structure of MRP1 is in part similar to P-gp. The studies suggest that unlike P-gp, the two NBF's in MRP1 are distinct and are non-interchangeable; however, as with P-gp, cooperativity between the two NBF's exists (Chang et *al.*, 1998; Nagata et *al.*, 2000). NBF1 in MRP1 has a higher affinity to bind to ATP, but its rate of ATP hydrolysis is low,

whereas NBF2 has a much lower affinity to bind to ATP but shows a higher catalytic rate (Hou et *al.*, 2002). Moreover, in MRP1 ATP bound to NBF1 triggers conformational changes within the protein that results in repetitive hydrolysis of ATP by NBF2. NBF2 is suggested to be the main ATP-binding site that controls MRP1 transport function (Haimeur et *al.*, 2004). It has been suggested that in MRP1 Pluronics® cannot fully access NBF2 and the high affinity drug binding sites (Batrakova et *al.*, 2004). This lack of accessibility could explain a relatively lower Pluronic® inhibitory effect on MRP1. Yet another explanation of this effect can be related to the activity of the GSH/GST detoxification system. It is well-known that many MRP1 substrates are GSH dependent, and GSH plays a significant role in MRP1-mediated transport (Hipfner et *al.*, 1999). A previous study on the function of MRP1 have shown a reduction in GSH intracellular levels and a decrease in GST activity in the presence of Pluronic® P85 (Batrakova et *al.*, 2003).

Unlike P-gp and MRP1 transporters, BCRP is a half transporter and contains one NBF, which precedes the six putative TM substrate-binding domains. BCRP must dimerize with another half transporter to form a functional transporter (Ozvegy *et al.*, 2001). In a study on BCRP overexpressing Baculovirus -transfected Sf9 insects cells, BCRP was shown to exhibit 2-3 fold higher ATPase activity than P-gp, when stimulated by the same substrate (Ozvegy *et al.*, 2001). Therefore, theoretically there may be several reasons why Pluronics® are unsuccessful at inhibiting BCRP-mediated drug resistance: 1) BCRP may not be sensitive to membrane fluidization by Pluronics® [and /or] 2) Pluronics® might be ineffective in the reduction of ATP, or in the inhibition of ATPase activity of BCRP [and /or] 3) Pluronics® may not be able to access and hinder the NBF, or the BCRP substrate binding sites when BCRP is dimerized with another half-transporter. More studies are certainly required to explain the lack of inhibitory activity of Pluronics® on BCRP.

Conclusion

Multidrug resistance caused by drug efflux, mediated by ABC proteins such as P-gp, MRP1 and BCRP, is a major obstacle that is often limiting the rate of success of chemotherapy treatment. Several modulators of the efflux systems have been previously tested to circumvent drug resistance; one group of them are Pluronic® block copolymers. In this study the activity of some Pluronics® as sensitizing agents and potential inhibitors of P-gp, MRP1 and for the first time, BCRP were investigated, and compared to some other known efflux inhibitors.

For each transporter, absorption and cytotoxicity studies were conducted. The accumulation studies were done to study the influence of Pluronics® on the efficacy of the efflux systems; cytotoxicity measurements were performed to study sensitization of cells to cytotoxic drugs by one polymer, Pluronic® L61.

The accumulation studies showed that P-gp expressing MCF-7 ADR cells were most responsive to Pluronics®. Hydrophobic polymers like Pluronics® L61, L92 and P123 at their maximum effective concentration increased the accumulation of R123 in MCF-7 ADR cells more than the less hydrophobic polymer Pluronic® P85. Pluronics® showed only limited effectiveness in inhibiting BCRP and MRP1-mediated efflux. Pluronics® L61 and P85 failed to increase the accumulation of TPT in BCRP overexpressing MCF-7 TPT cells. However, both polymers enhanced the accumulation of R123 in BCRP and MRP1 overexpressing cells. In our studies, the observed inhibitory effect of Pluronics® on HL 60 ADR cells could not be clearly attributed to MRP1 expression.

A key observation made during this study was related to substrate selectivity of P-gp, MRP1 and BCRP efflux systems inhibited by Pluronics®, and by other inhibitors. Pluronics® L61 and P85 have shown inhibitory effects by increasing the accumulation of all tested substrates: R123, Dox, MTX and TPT. However, the same polymers were only effective for the accumulation of R123 in MRP1 and BCRP overexpressing cells.

The sensitization effect of Pluronic® L61 was much greater for P-gp expressing MCF-7 ADR cells than on MRP1 expressing HL 60 ADR cells, and no sensitization was observed on BCRP overexpressing MCF-7 TPT cells.

To date, this is the first report investigating the effect of Pluronics® on BCRP mediated resistance. Even though our studies demonstrate no sensitizing effect of Pluronics® in BCRP overexpressing cells, further investigation are warranted to determine

the influence of Pluronics® on intracellular ATP levels, cell membrane viscosity, as well as substrate accumulation in various BCRP overexpressing cell types. Also, to further study the effects of Pluronic® and characterize the contribution of transporters on substrate accumulation, other studies using specific expression or inhibition (expression vector, siRNA) techniques should be performed. It would also be beneficial to obtain detailed proteomic and pharmacogenetic information to better understand the mechanism by which drug efflux proteins are able to bind and transport various substrates across cell membranes. Furthermore such studies could lead to discoveries of other human ABC proteins that might be involved in MDR, which haven't yet been identified.

In conclusion, Pluronics® can be very useful in the design of drug formulations, especially in overcoming drug resistance caused by P-gp. Supratek Pharma's SP1049C, a doxorubicin, and Pluronics® L61 and F127 formulation is an example of such a formulation, currently in phase II clinical trials.

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